In vitro studies on the mechanisms of action of chamomile, myrrh and coffee charcoal – components of a traditional herbal medicinal product (Myrrhinil-Intest®)

Der Fakultät für Biowissenschaften, Pharmazie und Psychologie

der Universität Leipzig

eingereichte

D I S S E R T A T I O N

zur Erlangung des akademischen Grades

doctor rerum naturalium

Dr. rer. nat.

vorgelegt von

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geboren am 15. Juli 1987 in Leipzig, Deutschland

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Tag der Verteidigung: 12. Dezember 2014

Bibliographic information

Vissiennon, C. J. R.: *In vitro* studies on the mechanisms of action of chamomile, myrrh and coffee charcoal – components of a traditional herbal medicinal product (Myrrhinil-Intest®)

University of Leipzig
Faculty of Bioscience, Pharmacy and Psychology, Institute of Pharmacy

Dissertation

128 pages, 62 figures, 31 tables

Abstract

The traditional herbal medicinal product Myrrhinil-Intest® is a fixed herbal combination, which is marketed in Germany since 1959 and applied in medical practice for the treatment of gastrointestinal disorders such as functional diarrhea, irritable bowel syndrome and inflammatory bowel disease. It contains myrrh, which is described as the oleo-gum resin from mainly *Commiphora molmol* Engler (Burseraceae), coffee charcoal, which are the milled roasted to blackening outer seed parts of green dried *Coffea Arabica* Linné (Rubiaceae) fruits and chamomile flowers - the flower heads of *Matricaria recutita* Linné (Asteraceae). The clinical effectiveness of Myrrhinil-Intest® for the treatment of various gastrointestinal disorders was demonstrated in several clinical studies and is described in various experience reports, however its pharmacological profile is not fully elucidated. Within the present study the spasmolytic and anti-inflammatory potential of the components myrrh, chamomile and coffee charcoal was investigated. Therefore pharmacological, histological and molecular biological methods were utilised. Spasmolytic activity was characterised using isometric tension measurement with rat isolated small intestinal preparations. Anti-inflammatory potential was assessed with different methods using isolated rat small intestinal preparations and immune cell lines. Inflammation was induced with TNBS and LPS respectively. Additionally, the influence of the herbal components on the gene expression profile of native human macrophages after LPS/IFNγ stimulation was determined by microarray gene expression analysis. Chamomile flower and myrrh exerted spasmolytic effects, whereby the more pronounced spasmolytic effects of myrrh were mediated via calcium channel blockade. Myrrh and chamomile flower exerted anti-inflammatory effects.

Die vorgelegte Arbeit wurde im Zeitraum vom 09.05.2012 bis zum 30.08.2014 an der Universität Leipzig, Fakultät für Biowissenschaften, Pharmazie und Psychologie, Institut für Pharmazie unter der Leitung von Frau Professorin Dr. sc. nat., habil. Karen Nieber angefertigt.

Selbstständigkeitserklärung

Hiermit versichere ich, dass die vorliegende Dissertation ohne unzulässige Hilfe und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde und dass die aus fremden Quellen direkt oder indirekt übernommenen Gedanken in der Dissertation als solche kenntlich gemacht worden sind.

Cica Vissiennon

Leipzig, den 25.09.2014

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List of abbreviations

ACh	acetylcholine
adjP	adjusted p-value
AG	aminoguanidine
ANOVA	analysis of variance
BH	Benjamini-Hochberg
BSA	bovine serum albumin
Bud	budesonide
cAMP-PDE	cAMP-phosphodiesterase
CC	coffee charcoal extract
cDNA	complementary deoxyribonucleic acid
COX-2	cyclooxygenase-2
CXCL10	chemokine receptor ligand 10
CXCL11	chemokine receptor ligand 11
CXCL12	chemokine receptor ligand 12
CXCL13	chemokine receptor ligand 13
DAD	diode array detector
DER	drug extract ratio
DMSO	dimethyl sulfoxide
e.g.	*exempli gratia,* for example
EC50	half maximal effective concentration
ELISA	enzyme-linked immunosorbent assay
Emax	maximum effect
EtOH	ethanol
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony stimulating factor
GO	gene ontological
HE	hematoxylin and eosin
HPLC	high-performance liquid chromatography
i.e.	*id est*
IBS	irritable bowel syndrome
IBS	inflammatory bowel disease
IC50	half maximal inhibitory concentration
IDO	indolamine 2,3-dioxygenase
IFNγ	Interferon-γ
IL-10	interleukin 10
IL-12	interleukin 12
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-8	interleukin 8
IRAK1	interleukin-1 receptor-associated kinase 1
IRAK4	interleukin-1 receptor-associated kinase 4
KA	chamomile flower extract
LAL	limulus amebocyte lysate
LDH	lactate dehydrogenase
LPS	lipopolysaccharides
MY	ethanolic myrrh extract
MYA	aqueous myrrh extract

NECA	5′-(N-ethylcarboxamido)adenosine
NO	nitric oxide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PI-IBS	post-infectious irritable bowel syndrome
PMA	phorbol-12-myristate-13-acetate
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RP	reversed phase
SEM	standard error of mean
SI	selectivity index
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNFα	tumor necrosis factor-α
TPA	12-O-tetradecanoylphorbol-13-acetate
u.c.	untreated control

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1 Introduction

1.1 Functional bowel disorders and irritable bowel syndrome

Functional bowel disorders are gastrointestinal disorders with symptoms attributable to the middle or lower gastrointestinal tract, which occur despite the absence of a structural or biochemical disorder. They include irritable bowel syndrome (IBS), functional bloating, functional constipation, functional diarrhea and unspecific bowel disorders (Longstreth *et al.* 2006).

Irritable bowel syndrome is one of the most common functional gastrointestinal disorders in humans (Cremonini and Talley 2005). It is characterised by abdominal pain and discomfort in association with altered bowel habits, whereby symptoms cannot be explained by any structural abnormalities (Drossman *et al.* 2002). Patients suffering from IBS can be classified based on the predominating stool pattern into symptom subgroups as diarrhea-predominant IBS, constipation-predominant IBS or IBS with alternating bowel movements (mixed IBS) (Longstreth *et al.* 2006).

Current evidence suggests a number of possible pathogenetical mechanisms including altered gastrointestinal motility, visceral hyperalgesia, post-inflammation alterations as well as psychosocial and environmental factors amongst others (Camilleri and Andresen 2009). Recent studies have focused on inflammatory processes as a key role in the pathophysiology of IBS. IBS patients show an increased number of T lymphocytes in intestinal tissue (Spiller *et al.* 2000), increased levels of pro-inflammatory cytokines such as TNFα, IL-8, IL-1β, IL-12 and IL-6 and lowered levels of anti-inflammatory cytokines including IL-10 (Bashashati *et al.* 2012). Gene polymorphism within the pro-inflammatory TNFα and anti-inflammatory IL-10 gene has been discussed as a risk factor for an altered immunological function in patients with IBS (Barkhordari *et al.* 2010). Furthermore, acute enteric infection has been shown to be an important risk factor for the development of IBS. So called post-infectious IBS (PI-IBS) occurs after an initial episode of acute gastrointestinal infection (Spiller and Campbell 2006). Patients with PI-IBS show a residual inflammation that gradually decreases, and persists for a year and longer (Scalera and Loguercio 2012).

In vivo studies indicated, that pain hypersensitivity and an enhanced colonic motility – characterised by increased phasic motility and enhanced smooth muscle tone – are independent factors contributing to symptom severity of IBS patients (Kanazawa *et al.* 2008). Intestinal hypermotility, amongst others, is therefore one target to control abdominal pain and diarrhea in IBS therapy which can be addressed by the use of antispasmodic and muscle relaxing agents.

However, traditional IBS therapies are mainly intended for the relief of individual symptoms and of limited efficacy in addressing the overall symptom complex (Camilleri and Andresen 2009).

In this context, the use of herbal medicinal products is a promising strategy in targeting the multifactorial pathophysiology of IBS, as they often provide a multi-target approach and a comparably low incidence for adverse events (Efferth and Koch 2011; Wagner 2006).

1.2 The herbal medicinal product Myrrhinil-Intest®

Myrrhinil-Intest® is the trade-mark name of a fixed combination consisting of myrrh, chamomile flower and coffee charcoal. It entered the market in Germany in 1959. Following the introduction of a national legal framework for medicines with the establishment of the German Medicines Act in 1976 (effective from 1st January 1978), it obtained a so-called subsequent approval by the Commission E in 2001. This subsequent approval was granted for medicines with in principle well-known constituents for which safety and efficacy was demonstrated by a scientific advisory board (German Commission E), whereby the manufacturer had to provide evidence for quality only. After the implementation of an European regulatory framework for herbal medicinal products in 2004 with the 'Directive on Traditional Herbal Medicinal Products' (Directive 2004/24/EC), which allows registration as a "traditional herbal medicinal product", the regulatory status of Myrrhinil-Intest® had to be adapted to a traditional use registration in 2008.

Myrrhinil-Intest® is indicated for the relief of gastrointestinal symptoms associated with functional gastrointestinal disorders. Its therapeutic use is mainly based on empiric knowledge by health care practitioners and focuses on the relief of symptoms associated with gastrointestinal disorders. The indication area is not clearly defined but several case reports and observational studies reflect the range of therapeutic indications and demonstrate clinical effectiveness.

In a study performed by Gruia for the treatment of gastroenteritis, gastroenteritis associated with diarrhea and enterocolitis, symptom relieve was observed after four weeks treatment with Myrrhinil-Intest® in the majority of patients (Gruia 1987). Beckmann *et al.* reported antimycotic effects *in vitro* which were confirmed in an *in vivo* case study by Lühr for the treatment of intestinal mycosis (Beckmann *et al.* 1996; Lühr 1996). A case report by Schinke suggests the use of Myrrhinil-Intest® in combination with products for intestinal sanitation for the treatment of chronic diarrhea (Schinke 2011). Stange *et al.* reported about a favourable development of a colitis ulcerosa case after Myrrhinil-Intest® administration as concomitant medication (Stange *et al.* 2004). Furthermore, Langhorst *et al.* performed a clinical trial over a 12 months period

for maintaining remission in ulcerative colitis. In this double-blind, double-dummy study, non-inferiority was found for the treatment with Myrrhinil-Intest® against mesalazine (Langhorst *et al.* 2013).

It becomes apparent from the clinical reports that the therapeutic area for which Myrrhinil-Intest® is applied, covers various gastrointestinal disorders such as intestinal mycosis, chronic diarrhea and inflammatory bowel disease as well as irritable bowel syndrome.

1.2.1 Myrrh, *Commiphora molmol*

Myrrh is described as the oleo-gum resin from mainly *Commiphora molmol* Engler (Burseraceae). The resin is obtained by incision or produced by spontaneous exudation from stem and branches of *Commiphora* species (Myrrha. European pharmacopoeia 2008). The reddish-brown resinous exudate has a bitter and acrid taste and a balsamic odour (Evans and Trease 1989).

Myrrh consists mainly of volatile oil (3-8 %), alcohol-soluble resin (25-40 %) and water-soluble gum (30-60 %) (Tucker 1986). The main constituents of the volatile oil are furano-sesquiterpenes, while the resin is comprised of di- and triterpenes. The water-soluble gum fraction consists of a heterodispers mixture of proteoglycans (Hanus *et al.* 2005; Wiendl *et al.* 1995).

The medicinal usage of myrrh dates back to biblical times and has been mentioned in both the Bible and the Koran as well as in ancient literature of Rome and Greece (Madaus 1938). Its traditional uses cover treatment of wounds, pain, arthritis, fractures, obesity, parasitic infection and gastrointestinal disorders (Shen *et al.* 2012). The German Commission E recommends the external use of myrrh for topical treatment of mild inflammations of the oral and pharyngeal mucosa, because of its adstringent properties (Bundesinstitut für Arzneimittel 15.10.1987).

1.2.2 Chamomile, *Matricaria recutita*

Chamomile flowers are the dried flower heads of *Matricaria recutita* L. (Asteraceae) indigenous to the Eastern Mediterranean region. The flowers have a pleasant sweet-herbaceous and fruity odour and an aromatic, slightly bitter taste (Hänsel and Sticher 2010). Main constituents of chamomile flowers are essential oil (0.4 – 2.0 %) composed of chamazulene and α-bisabolol, amongst flavonoids (up to 8 %) with mainly apigenin, quercetin and luteolin. Other constituents include polysaccharides, lipids and coumarins (Maha Aboul Ela 2012)

Chamomile has been used for centuries as a medicinal plant because of its well-known anti-inflammatory, antioxidant and mild adstringent properties (Gupta 2010; Weiss and Fintelmann 2000).

The anti-inflammatory potential of chamomile flowers is well known and has been confirmed in several animal models such as the carrageenan-induced rat paw oedema model (Al-Hindawi *et al.* 1989; Gerritsen *et al.* 1995) or the croton oil induced oedema model (Della Loggia *et al.* 1990; Tubaro *et al.* 1984). Furthermore, beneficiary effects of chamomile-containing rinses for the treatment of oral mucositis or stomatitis induced by cancer therapies as well as the topical treatment on inflammations of the skin was demonstrated in several clinical studies (Aertgeerts *et al.* 1985; Carl and Emrich 1991; Fidler *et al.* 1996; Patzelt-Wenczler and Ponce-Pöschl 2000).

The spasmolytic activity of chamomile flowers has been investigated in several animal models. Antispasmodic effects of the chamomile component (-)-α-bisabolol have been reported by Hava and Janku already in 1957. Forster *et al.* further demonstrated the inhibitory activity of an ethanolic chamomile extract (31 % v/v ethanol, DER: 1:3.5) on acetylcholine- and histamine-induced contractions in isolated guinea pig ileum (Forster *et al.* 1980). Furthermore, Achterrath-Tuckermann *et al.* found that especially α-bisabolol and to a fewer extent the bisabololoxides A and B are responsible for the spasmolytic activity (Achterrath-Tuckermann *et al.* 1980). A positive monograph of the German Commission E recommends the internal use of chamomile flowers as oral infusion for the treatment of gastrointestinal spasms and inflammatory diseases of the gastrointestinal tract. External use as rinses or bath additive is recommended for the treatment of skin and mucous membrane inflammations (Bundesinstitut für Arzneimittel 05.12.1984).

1.2.3 Coffee charcoal, *Coffea Arabica*

Coffee charcoal is described as the milled, roasted to blackening outer seed parts of green dried *Coffea Arabica* L. fruits (Rubiaceae). August G. Heisler introduced coffee charcoal into medical practice around 1937 for the treatment of various diseases such as angina, scarlet fever, parodontosis but also acute and chronic gastrointestinal disorders. The term coffee *charcoal* might be misleading as it must not be understood as a carbonised product such as activated charcoal (*carbo medicinalis*), but overroasted coffee, which retains its aroma and most of its constituents (Kuhn and Schäfer 1939).

The main constituents of the unroasted beans (green coffee) are purine alkaloids with mainly caffeine, the nicotinic acid derivative trigonelline, acids and esters including chlorogenic acids and aliphatic acids, lipids including coffee oil and diterpenes (Clarke 2003; Trugo 2003). The seed composition changes during the roast process due to pyrolysis, caramelisation and Maillard reactions. The lipid fraction is relatively heat stable and also the caffeine content is not markedly altered during roasting. However, chlorogenic acids undergo many changes and due to thermal instability degradation occurs upon drastic roasting conditions (Farah and

Donangelo 2006). Roasting furthermore degrades trigonelline, producing a variety of compounds including nicotinic acid and volatile compounds such as pyrrols, pyridines and methyl nicotinate (Trugo 2003).

The main constituents of coffee charcoal are similar to those of roasted coffee, however the quantities differ due to the longer roasting time. Kuhn and Schäfer have analysed the ingredients of coffee charcoal and found that the content of caffeine and chlorogenic- and caffeic acid is not markedly reduced and coffee charcoal contains 75 % of the caffeine content in roasted coffee. However, it contains only half of the trigonelline content of roasted coffee (Kuhn and Schäfer 1939).

Coffee charcoal is marketed in Germany since many decades under the trade name “Carbo Königsfeld®” also known as “Heislersche Kaffeekohle” for the treatment of unspecific acute diarrhea. The medicinal use of coffee charcoal (*coffea carbo*) is recommended by the German Commission E for the treatment of unspecific, acute diarrhea and local therapy of mild inflammation of the oral pharyngeal mucosa because of its absorbent and adstringent properties (Bundesinstitut für Arzneimittel 05.05.1988).

1.3 Motivation and thesis objective

Following the establishment of the German Medicines Act in 1976, which introduced a definition of medicinal products, manufacturers were obliged to provide proof of quality for the medicinal product, whereby safety and efficacy for in principle well-known constituents of herbal medicinal products could be demonstrated by the Commission E. Myrrhinil-Intest® obtained a subsequent approval within this regulatory context in 2001.

In the course of further harmonisation across the European Union the Directive on Traditional Herbal Medicinal Products (Directive 2004/24/EC) introduced a regulatory framework for herbal medicinal products which allows a registration as “traditional herbal medicinal product” or marketing authorisation for well-established medicinal products. Within this context, the regulatory status for Myrrhinil-Intest® had to be adapted to a traditional use registration and new documentation in line with the requirements from the directive (Directive 2004/24/EC § 16a) has to be provided. One aspect within these requirements concerning pharmacological proof, asks for "plausibility of pharmacological effects or efficacy of the medicinal product on the basis of long-standing use and experience" (Directive 2004/24/EC § 16a).

Alongside with these increasing regulatory requirements for herbal medicinal products, companies were obliged to provide, *inter alia*, solid data regarding efficacy of their medicinal product. This knowledge has to be reinforced with the provision of robust preclinical data.

Therefore, detailed information about molecular and functional effects *in vitro* as well as *in vivo* are required to elucidate underlying mechanisms, which could contribute to the plausibility of clinical effectiveness. To address the increasing need for robust preclinical data, the pharmacological profile of Myrrhinil-Intest® has to be updated with the means of modern biochemical and pharmacological methods.

Looking at the spectrum of therapeutic indications which have emerged during the past 50 years, mainly spasmolytic and anti-inflammatory effects seem to contribute to the reported therapeutic effectiveness.

For the treatment of inflammatory intestinal disorders such as inflammatory bowel disease and irritable bowel syndrome, intestinal hypermotility is a promising target to control abdominal pain and diarrhea. In this context, spasmolytic activities besides anti-inflammatory effects are of interest to target inflammation-induced contractile disturbances.

Especially the anti-inflammatory and spasmolytic effects of chamomile are well-established and known for centuries. It is however necessary to obtain a complete picture of the pharmacological profile of the specific plant preparations used in Myrrhinil-Intest®.

In this context the objective of the investigations were to elucidate the following questions:

Are pharmacological effects such as spasmolytic and anti-inflammatory activities of the single components of Myrrhinil-Intest® (myrrh, chamomile flower and coffee charcoal) detectable, which provide a rational for the treatment of inflammatory intestinal disorders?

What mechanisms could be underlying the pharmacological activities?

In order to examine these issues, the aims of the present thesis were in particular:

i. Investigation of spasmolytic activities of the myrrh, chamomile and coffee charcoal preparations. These should be examined using isometric tension measurement of isolated rat small intestinal preparations. Furthermore, spasmolytic activities should be revised under inflammatory conditions since gastrointestinal disorders are often accompanied by inflammatory processes and alterations in morphology and motility of intestinal tissue have been reported (Poli *et al.* 2001).

ii. Investigation of anti-inflammatory activities of the myrrh, chamomile and coffee charcoal preparations. For the examination an *in vitro* model for acute inflammation of rat small intestinal preparations should be used. Estimation of the inflammatory damage should be based on functional performance using isometric tension measurement, morphology using histological analysis and expression of TNFα-mRNA using quantitative PCR.

iii. Investigation of anti-inflammatory activities of the myrrh, chamomile and coffee charcoal preparations using cell culture methods. In particular it should be examined, whether the components influence cytokine signalling of immune cells.

iv. Determination of mechanisms underlying potential anti-inflammatory activities of the components, using gene expression analysis in native human macrophages.

2 Material and methods

2.1 Preparation of plant material

The herbal medicinal product Myrrhinil-Intest® is a combination product consisting of chamomile flower extract, powdered myrrh and powdered coffee charcoal. For pharmacological testing, suitable formulations needed to be established out of the original plant material which was used for the manufacturing process. Therefore, aqueous and/or ethanolic extracts were produced if necessary.

2.1.1 Powdered myrrh

Powdered myrrh as source material, compliant with specifications in the European Pharmacopoeia monograph (Myrrha. European pharmacopoeia 2008), was provided by Lomapharm, Emmerthal, Germany (Myrrhe Gum EB/BP pulv., batch-no. JA0167, Lomapharm, Emmerthal, Germany). For the pharmacological testing, an aqueous and an ethanolic extract was prepared. Figure 1 shows a schematic outline of the extraction processes.

For the preparation of the aqueous myrrh extract (MYA), 1.0 g powdered myrrh was extracted three times with ethanol (30 ml; 96 % v/v) for 30 minutes. The residue was then extracted exhaustively with purified water (first extraction: 40 ml purified water for 15 min; second and third extraction: 15 ml purified water for 10 minutes). The residue was removed and the combined supernatants were freeze dried. The lyophilisate was stored at -20°C until further use.

An ethanolic myrrh extract (MY) was kindly provided by D&S Pharma GmbH (batch-no. 18719, 01/2012, Ibbenbüren, Germany). For preparation of the dried extract, powdered myrrh was extracted under reflux with ethanol (96 % v/v) at 90°C for 60 minutes. After evaporation to dryness the residue was suspended in water and steam distillation was performed. The dried residue was stored at -20°C until further use.

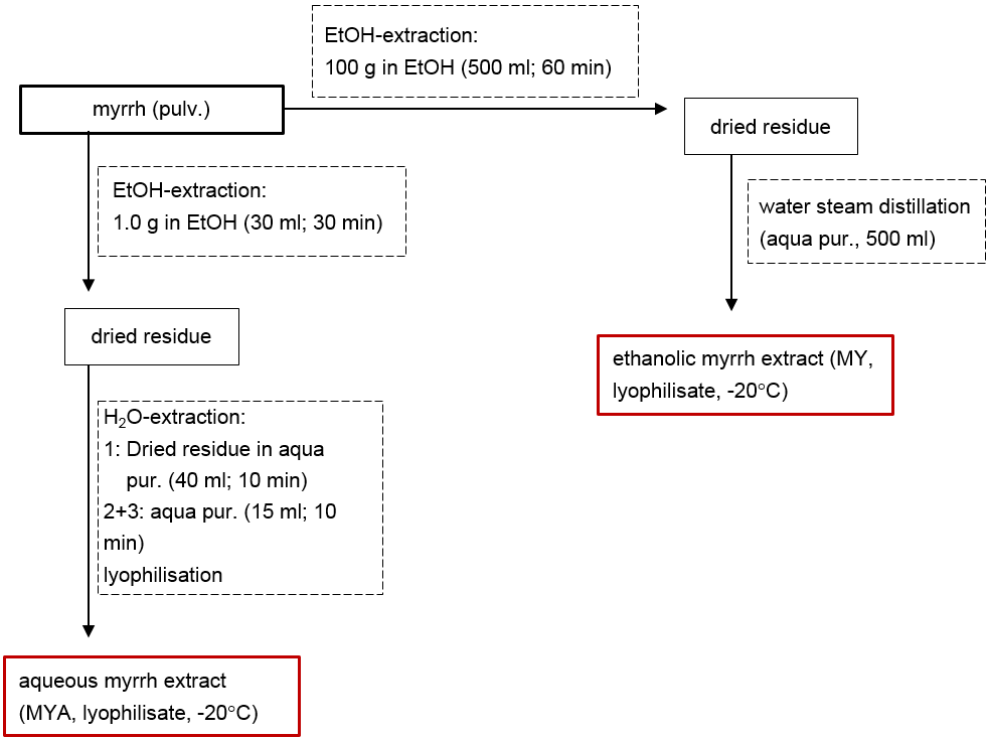

Figure 1: Schematic outline of the extraction process of powdered myrrh.
For the preparation of the aqueous myrrh extract (MYA), the powdered substance was extracted with ethanol followed by an exhaustive water extraction and lyophilisation process. The ethanolic myrrh (MY) extract was obtained by ethanolic extraction and subsequent water steam distillation.

Characterisation of the ethanolic myrrh extract HPLC analysis was performed by D&S Pharma GmbH (Ibbenbüren, Germany) using a Multosper 100 RP 18 column (250 mm x 4 mm; 5 µm) and a DAD detector (5 cm). The mobile phase consisted of 39 % (v/v) acetonitrile, 60 % (v/v) water and 1 % (v/v) acetic acid (I) and 99 % (v/v) water and 1 % (v/v) acetic acid (II) at a flow rate of 1.0 ml/min. A gradient program was used as follows: 0 min, 100 % (I); 30 min, 100 % (I); 70 min, 90 % (I) and 10 % (II); 80 min, 100 % (I). The monitor wave length of the DAD detector was 240 nm. The HPLC fingerprint of ethanolic myrrh extract is presented in Figure 2.

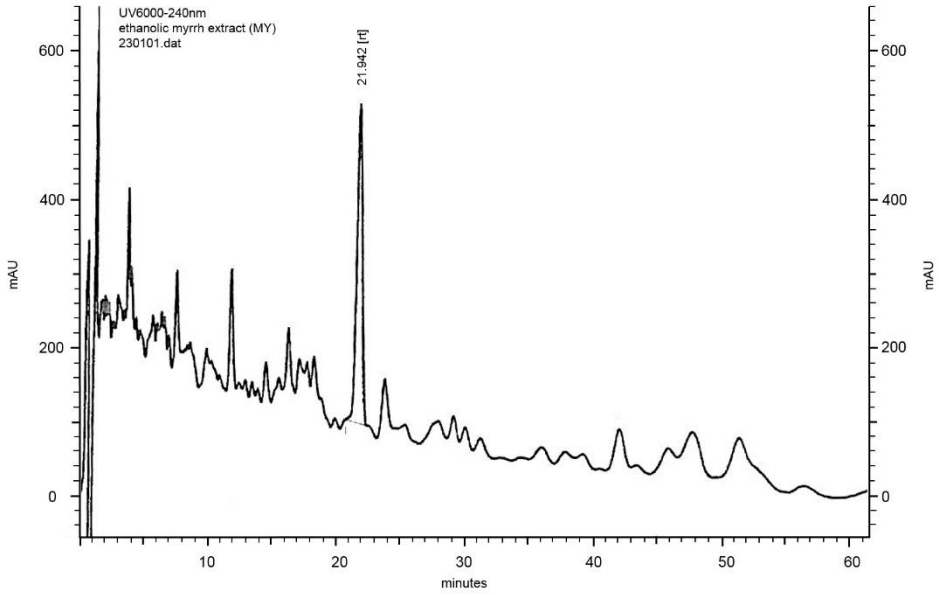

Figure 2: HPLC-fingerprint of the ethanolic myrrh extract (MY).
HPLC analysis was performed using a Multosper 100 RP 18 column (250 mm x 4 mm; 5 µm) and a DAD detector (5 cm) with a mobile phase consisting of 39 % (v/v) acetonitrile, 60 % (v/v) water and 1 % (v/v) acetic acid (I) and 99 % (v/v) water and 1 % (v/v) acetic acid (II) at a flow rate of 1.0 ml/min. Kindly provided by D&S Pharma GmbH, Ibbenbüren, Germany.

2.1.2 Chamomile flower extract

The chamomile flower dry extract (EtOH 60 % m/m; DER: 4-6:1; batch-no. HC0070) used in the finished dosage form was provided by Lomapharm, Emmerthal, Germany.

2.1.3 Coffee charcoal

Powdered coffee charcoal was provided by Lomapharm (Carbo Coffea EB 6, batch-no. JB0142, Lomapharm, Emmerthal, Germany). For the preparation of an aqueous extract, 20.0 g powdered substance were extracted in 150 ml boiling purified water for 15 minutes. After filtration the filtrate was freeze dried and the lyophilisate was stored at -20°C until further use.

2.1.4 Determination of endotoxin contamination

All plant extracts were tested for endotoxin contamination since especially cell biological experiments using immune cells react very sensitive to traces of endotoxins which could distort the results. Therefore, the ToxinSensor Gel Clot Endotoxin Assay Kit (GenScript, NJ, USA) was used according to manufacturer's instructions. This Limulus Amebocyte Lysate (LAL) test is based on the endotoxin-induced coagulation of enzymes in blood cells (amebocytes) of the horseshoe crab *(Limulus polyphemus)* (Levin and Bang 1964). In this study a gel clotting assay was preferred instead of a colorimetric assay, since the self-coloration of the extracts could distort the interpretation of the results. The test was performed by mixing 100 µl of the reconstituted lyophilised Limulus Amebocyte Lysate with 100 µl extract dilutions (1.25 – 5 x 10^{-6} g/ml). E.coli endotoxin standard and endotoxin-free water (both provided in kit) were used as positive and negative control respectively. After 60 minutes incubation at 37°C the samples were analysed visually for gel clotting.

The test results for all plant extracts were negative in the concentrations tested, which means that the endotoxin level in all extracts is below the detection limit of 0.25 endotoxin units (EU)/ml corresponding to 25 pg/ml.

2.1.5 Materials and equipment

Table 1: Preparation of plant materials – Substances and consumables

Substance/consumable	**Supplier**
Myrrhe Gum pulv., (batch-no. JA0167)	Lomapharm, Emmerthal, Germany
Chamomile flower dry extract (EtOH 60 % m/m; DER: 4-6:1; batch-no. HC0070)	Lomapharm, Emmerthal, Germany
Carbo Coffea EB 6 (batch-no. JB0142)	Lomapharm, Emmerthal, Germany
Ethanol, absolute	Grüssing GmbH, Filsum, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
ToxinSensor Gel Clot Endotoxin Assay Kit	GenScript, NJ, USA

Table 2: Preparation of plant materials – Equipment

Equipment	**Manufacturer**
Lyophilisator	Christ, Osterode, Germany

2.2 Animals and tissue preparation

Adult male and female Wistar rats (*Rattus norvegicus*, var. albinos; 12-17 weeks old, 250 to 500 g body weight) were purchased from Biomedical Centre, Medical Faculty, University of Leipzig, Germany. The animals were housed in cages of five at room temperature in a 12 hour light/dark cycle. Tap water and standard food pellets were available *ad libitum*. All experiments were performed according to the German Animal Welfare Act and approved by the Institutional Review Board of Animal Care Committee (reference number: T 06/13, date of approval: 12th November 2012).

Isolation of the ileum/jejunum preparations was performed after the rats were anaesthetised with CO_2 and sacrificed by decapitation. Subsequently, the abdomen was opened immediately by midline incision and the distal segment of the small intestine of approximately 15 cm was excised and stored in aerated modified Krebs solution at 37°C.

2.3 Induction of inflammation – TNBS-inflammation model

Inflammation was induced by instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) into the lumen of the ileum/jejunum preparations (TNBS-inflammation model acc. to Warstat 2004). Thus, an approximately 2.5 cm long ileum/jejunum preparation was prepared and cleaned. One end was closed by a thread whereas a cannula was installed in the other end through which TNBS (10-100 mM) or modified Krebs solution was applied (Figure 3). After application, the cannula was removed and the end was closed by a thread. Depending on the respective objective, the preparations were then treated accordingly:

For measurement of isometric contractions (see 2.4) the preparations were incubated with TNBS (10 mM) or the plant extracts in different concentrations together with TNBS (10 mM) for 30 minutes.

For histological analysis (see 2.5), the preparations were incubated with TNBS (100 mM) or the plant extracts in different concentrations together with TNBS (100 mM) for 30 minutes.

For determination of TNFα gene expression (see 2.6) the preparations were incubated with TNBS (10 mM) or the plant extracts in different concentrations together with TNBS (10 mM) for 3 hours.

In all experiments, preparations incubated with TNBS or untreated preparations served as positive and untreated controls respectively.

The incubations were carried out in a 10 ml incubation chamber containing aerated (95 % O_2, 5 % CO_2) modified Krebs solution. At the end of incubation time, the threads were removed and the preparations rinsed with modified Krebs solution. Sections of 1.5 cm (measurement of

isometric contractions) or 0.5 cm (histological analysis and TNFα gene expression analysis) length were prepared for the experiments.

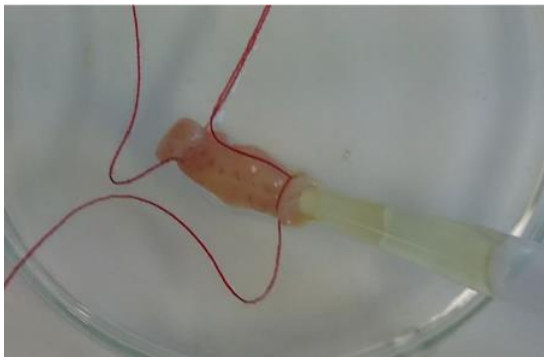

Figure 3: Induction of inflammation
For induction of inflammation a rat ileum/jejunum preparation (2.5 cm) was cleaned and 2,4,6-trinitrobenzenesulfonic acid (TNBS, 10 mM) was applied through a cannula and incubated for 30 minutes.

2.4 Measurement of isometric contractions

Isometric contraction were measured using an organ bath equipment (TSE Systems, Bad Homburg, Germany). The equipment consists of 4 separate organ baths.

The ileum/jejunum preparations were obtained and treated as outlined in '2.2 Animals and tissue preparation' and '2.3 Induction of inflammation – TNBS-inflammation model' and suspended in 20 ml organ baths containing aerated (95 % O_2, 5 % CO_2) modified Krebs solution maintained at 37°C.

Using polyester threads, one end was anchored to a stationary hook at the bottom and attached to an isometric transducer (TSE Systems, Bad Homburg, Germany) for continuous recording of the isometric tension. The preparations were allowed to equilibrate for 40 minutes with a preloaded tension of 10 mN.

Acetylcholine (ACh, 1 mM) was applied directly into the organ baths to induce tonic contraction. After reaching the force maximum the organ baths were rinsed three times with modified Krebs solution to remove all applied substances from tissue and bath vessels.

2.4.1 Test execution

Depending on the issue in question, different experimental designs were carried out.

In order to examine the influence on ACh-induced contraction, the following experiment set up was used:

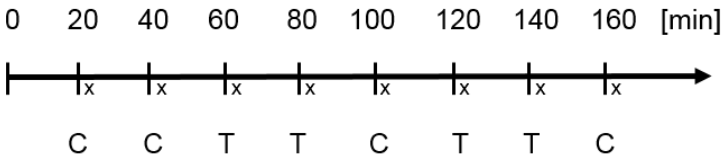

Figure 4: Experimental set up for investigation of influence on ACh-induced contraction.
C – Control application of ACh (internal standard); T – Application of test substance two minutes prior to ACh application; x – wash out.

An initial ACh (1 mM) application at minute 20 was performed for conditioning and to test the sensitivity of the preparations used. Further single ACh applications (minute: 40, 100 and 160) were used as internal standards. Between these control applications, the influence of test substances was examined by application two minutes prior to ACh application (minute: 60, 80, 120 and 140).

To assess the influence on TNBS-induced contractility decrease, the following experimental set up was used:

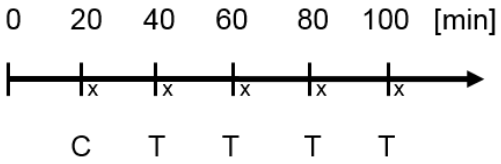

Figure 5: Experimental set up for investigation of influence on TNBS-induced contractility decrease.
C – Control application of ACh (internal standard); T – Application of ACh; x – wash out.

After the initial ACh (1 mM) application at minute 20, ACh (1 mM) applications and subsequent wash out period were carried out every 20 minutes.

For the characterisation of calcium antagonistic effects, the following experiment set up was applied:

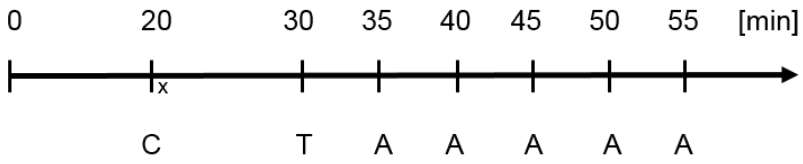

Figure 6: Experimental set up for the characterisation of calcium antagonistic effects.
C – Control application of ACh; T – Application of test substance; A – Application of calcium channel agonist Bay K8644; x – wash out.

An initial ACh (1 mM) application at minute 20 was performed to test sensitivity of the preparations used. Afterwards, the preparations were pre-treated with the test substance at minute 30. Cumulative applications of L-type calcium channel agonist BAY K8644 (10^{-10} – 10^{-5} M) were subsequently conducted every 5 minutes.

2.4.2 Data analysis

For data acquisition, BioSys software (TSE Systems, Bad Homburg) was used recording ten measured values (force in mN) per second. Further data analysis was performed using Microsoft Excel (version 14.0, Microsoft cooperation, Redmond, WA, USA) and Graph Pad Prism (version 6.00, GraphPad Software Inc., San Diego, CA, USA) for statistical analysis.

ACh-induced contractions were expressed as % of the contraction peak induced by a control application (minute 40). Basal muscular tone was expressed as mean tension over a 60 second time period compared to an initial control period (= 100 %). Data are expressed as mean ± SEM; n represents the number of different animals used.

Statistical analysis of ACh-induced contraction was performed by one-way analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test. For the characterisation of calcium antagonistic effects, concentration-response curves and corresponding parameters such as EC/IC50 and Emax were obtained using non-linear regression by GraphPad Prism software. Comparison of the parameters EC50 and Emax of the agonistic concentration-response curves was done by comparison of fits. Comparison of EC50 values was performed using paired, two-tailed t-test.

Schild analysis for the calculation of pA_2 and pD_2 values was performed according to the following equations:

$$I: \quad pA_2 = -\log[B] + \log\frac{EC50_A}{EC50_{AB}} - 1$$

$$II: \quad pD_2 = -\log[B] + \log\frac{E_A}{E_{AB}} - 1$$

[B] – antagonist concentration
$EC50_A$ – EC50 value, agonist only
$EC50_{AB}$ – EC50 value, after pre-treatment with antagonist;
E_A – maximum effect, agonist only
E_{AB} – maximum effect, after pre-treatment with antagonist

2.4.3 Materials and equipment

Table 3: Isometric tension measurement – Substances and consumables

Substance/consumable	**Supplier**
Bay K8644	Sigma-Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
Acetylcholine (ACh)	Sigma-Aldrich, Steinheim, Germany
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	Sigma-Aldrich, Steinheim, Germany
CO_2	Air Liquide, Leipzig, Germany
Carbogen (95 % O_2, 5 % CO_2)	Air Liquide, Leipzig, Germany

Table 4: Isometric tension measurement – Substances and consumables (Modified Krebs solution acc. to Martinsson)

Substance/consumable	**Concentration**	**Supplier**
Modified Krebs solution acc. to Martinsson, pH 7.4		
Sodium chloride (NaCl)	118 mM	AppliChem, Darmstadt, Germany
Sodium bicarbonate ($NaHCO_3$)	25 mM	Sigma-Aldrich, Steinheim, Germany
Potassium chloride (KCl)	4,8 mM	Sigma-Aldrich, Steinheim, Germany
Magnesium sulfate heptahydrate ($MgSO_4 * 7H_2O$)	1,2 mM	Fluka, Buchs, Schweiz
Monopotassium phosphate (KH_2PO_4)	1,2 mM	Fluka, Buchs, Schweiz
Calcium chloride dihydrate ($CaCl_2 * 2\ H_2O$)	2,5 mM	Sigma-Aldrich, Steinheim, Germany
Glucose	11 mM	Sigma-Aldrich, Steinheim, Germany

Table 5: Isometric tension measurement – Equipment

Equipment	**Manufacturer**
Organ bath equipment	TSE Systems, Bad Homburg, Germany
Hand lever guillotine	Hugo Sachs, March-Hugstetten, Germany

2.5 Histological analysis

2.5.1 Fixation, processing, embedding and sectioning of samples for histological analysis

For histological analysis, ileum preparation were prepared and processed as outlined in '2.2 Animals and tissue preparation' and '2.3 Induction of inflammation – TNBS-inflammation model'. Morphological variance was kept at a minimum by using particularly the ileum segment of the small intestine (4-5 cm).

After incubation, the preparations were fixated for at least 24 hours in formalin (4 % formaldehyde in phosphate buffered saline; pH 7.4) at room temperature and rinsed with phosphate buffered saline (PBS, pH 7.4) followed by an initial 60 minute dehydration process with 50 and 70 % ethanol (v/v). The fixated preparations were subsequently dehydrated and infiltrated with paraffin wax using an automated tissue processor (Leica ASP200S; Leica Microsystems; Wetzlar Germany). A schematic representation of the embedding process is shown in Figure 7.

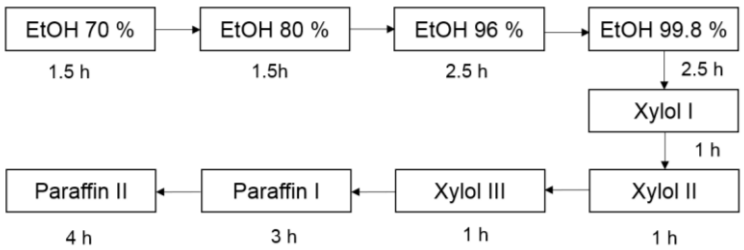

Figure 7: Schematic embedding process
After dehydration with increasing ethanol concentrations and xylol treatment preparations were infiltrated and stored in paraffin (59°C).

2.5.2 Preparation and staining of paraffin sections

After paraffin infiltration, the preparations were stored in liquid paraffin at 59°C and embedded in paraffin blocks using an embedding machine (Leica EG1160 Leica Microsystems, Wetzlar, Germany). After cooling and hardening of the paraffin blocks, a rotary microtome (Leica RM2255, Leica Microsystems, Wetzlar Germany) was used to prepare slices of 7 µm layer thickness which were placed on a microscope slide.

2.5.3 Hematoxylin and eosin (HE) staining

Hematoxylin and eosin (HE) staining is a routine staining method to visualise tissue morphology. The process uses a haematoxylin dye to stain basophilic structures like cell nuclei blue and an eosin dye to stain acidophilic structures like cytoplasm and cell walls pink or red.

Before staining, the slices were deparaffinised using xylol and ethanol (99.8 – 70 % v/v). After rehydration, staining with hematoxylin (Hematoxylin-solution, Mayer's, Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany) and eosin solution (0.1 %) was performed followed by a dehydration process using ethanol (99.8 % v/v) and xylol. The complete staining procedure is presented in Figure 8. For the preparation of durable samples slides were mounted with Canada Balsam (Dr. K. Holborn, Leipzig, Germany).

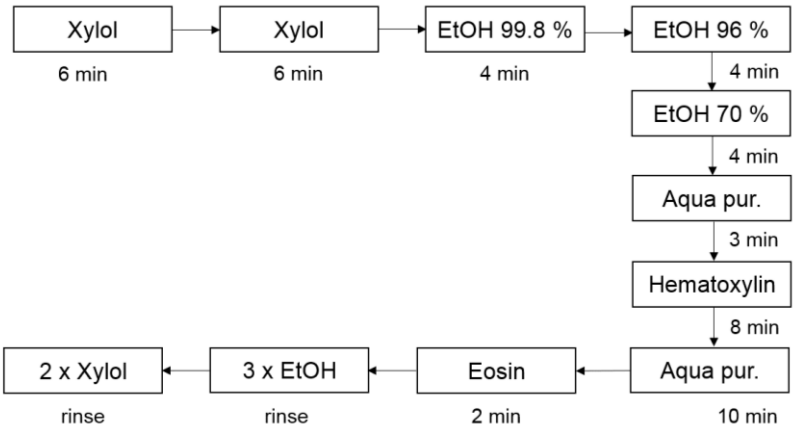

Figure 8: HE-staining protocol
After rehydration using xylol and decreasing ethanol concentrations, preparations were stained with hematoxylin and eosin followed by a dehydration process using ethanol and xylol.

2.5.4 Data analysis

After hardening of the mounting medium, morphometric analysis was performed using a transmitted-light microscope (Leica DMRBE, Leica Wetzlar Germany). Mucosa layer thickness was determined using ImageJ software (version 1.47 Wayne Rashband, National Institute of Health, USA) in three replicates within one sample and mean values were evaluated (Figure 9). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test using Graph Pad Prism software (version 6.00, GraphPad Software Inc., San Diego, CA, USA). Mucosa layer thickness of untreated preparations served as control (100 %). Data are expressed as mean ± SEM; n represents the number of different animals used.

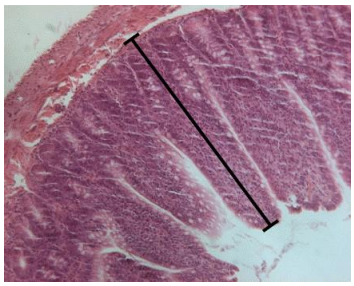

Figure 9: Morphometric analysis of rat ileum preparations
Mucosa layer thickness [µm] was measured using ImageJ software (version 1.47 Wayne Rashband, National Institute of Health, USA).

2.5.5 Materials and equipment

Table 6: Histological analysis - Substances and consumables – formalin 4 %; pH 7.4

Substance	Amount	Supplier
Paraformaldehyde	4.0 g	Sigma-Aldrich, Steinheim, Germany
Monosodium phosphate (NaH_2PO_4)	0.96 g	Sigma-Aldrich, Steinheim, Germany
Disodium phosphate (Na_2HPO_4)	0.284 g	Sigma-Aldrich, Steinheim, Germany
Aqua purificata	100 ml	

Table 7: Histological analysis - Substances and consumables – phosphate buffered saline (PBS); pH 7.4 acc. to Sørensen

Substance	Amount	Supplier
Monopotassium phosphate (KH_2PO_4)	2.722	Fluka, Buchs, Schweiz
Disodium phosphate (Na_2HPO_4)	11.36 g	Sigma-Aldrich, Steinheim, Germany
Aqua purificata	1000 ml	

Table 8: Histological analysis - Substances and consumables – Eosin solution

Substance	Amount	Supplier
Eosin	0.1 g	Sigma-Aldrich, Steinheim, Germany
Acetic acid	1 gt	
Aqua purificata	100 ml	

Table 9: Histological analysis - substances and consumables

Substance	Supplier
Hematoxylin-solution, Mayer's	Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany
Canada Balsam	Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany

Table 10: Histological analysis - equipment

Equipment	Manufacturer
Automated tissue processor (Leica ASP200S)	Leica Microsystems, Wetzlar, Germany
Embedding machine (Leica EG1160)	Leica Microsystems, Wetzlar, Germany
Rotary microtome (Leica RM2255)	Leica Microsystems, Wetzlar, Germany
Transmitted-light microscope (Leica DMRBE)	Leica Microsystems, Wetzlar, Germany

2.6 TNFα gene expression analysis in ileum/jejunum preparations

2.6.1 RNA-preparation and reverse transcription

The ileum/jejunum preparations were obtained and treated as described in 2.2 'Animals and tissue preparation' and 2.3 'Induction of inflammation – TNBS-inflammation model'. After incubation, tissue segments of approximately 0.5 cm length were obtained and total RNA was isolated using peqGOLD RNAPure (PEQLAB Ltd., Erlangen, Germany) according to manufacturer's instructions. Thus, samples were incubated for five minutes in 1 ml peqGOLD RNA Pure (phenol and guanidinium thiocyanate) at room temperature. After incubation 200 µl chloroform were added and the mixture was shaken for 15 seconds and placed on ice for 10 minutes. After phase separation and centrifugation, the aqueous phase including dissolved RNA was removed and the same volume of isopropyl alcohol was added. After 15 minutes incubation on ice the precipitated RNA was centrifuged and supernatant was removed. After two washing steps with ethanol (70 % v/v) the RNA pellet was dissolved in 30 µl nuclease-free water.

For cDNA synthesis oligo(dT)-primed reverse transcriptase was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to manufacturer's instructions. Thus, aliquots of 10 µg RNA were mixed with 1 µl Oligo$(dT)_{18}$ Primer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and incubated for 5 minutes at 70°C (MJMini™ Personal Thermal Cycler) followed by an incubation on ice for 2 minutes. A master

mix consisting of the following components was then added to each tube: 4 µl 5x first-strand buffer, 1.5 µl nuclease-free water, 0.5 µl RiboLock RNase Inhibitor, 2 µl desoxyribonucleotide triphosphate mix (dNTP mix, 10 mM each dATP, dGTP, dCTP, dTTP in DNase/RNase ultrapure water) and 1 µl RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA). The mixture was then incubated at 42°C for 60 minutes followed by an inactivation step at 70°C for 10 min.

RNA and cDNA quantity and purity were assessed using NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Nucleic acid purity was determined by the ratio of absorbance at 260 nm to 280 nm (approximately 1.8 for DNA; approximately 2.0 for RNA) and at 260 and 230 (between 1.8 and 2.2). cDNA samples were stored at -20°C until further use.

2.6.2 Quantitative real-time PCR (qPCR)

Quantitative determination of TNFα-gene expression was performed by quantitative real-time PCR on a MyIQ™ Cycler (Bio-Rad, Munich, Germany) using KAPA™ SYBR® FAST, qPCR Universal MasterMix (PEQLAB Ltd., Erlangen, Germany) according to manufacturer's instructions. The applied reaction set up for 15 µl total reaction volume is specified in Table 11. All qPCR reactions were run in duplicates and amplified using the amplification program specified in Table 12. Melt curve analysis was performed after the last cycle, to monitor the assay's specificity, whereby a single melt curve for each amplicon was required for specificity validation.

As reference gene '18S' ribosomal RNA was utilised as endogenous control for relative quantification of the target gene.

Table 11: qPCR Reaction set up

Component	**Volume**
KAPA™ SYBR® FAST, qPCR MasterMix (2x)	7.5 µl
Forward Primer (5 µM)	1.5
Reverse Primer (5 µM)	1.5
cDNA template	1 µl
Water, nuclease-free	3.5

Table 12: qPCR amplification program and melt curve analysis

Step	Temperature	Duration	Cycles
KAPA™ SYBR® DNA polymerase activation	95°C	10 min	1 x
Denaturation	95°C	15 sec	40 x
Annealing	60°C	30 sec	
Elongation	72°C	30 sec	
Dissociation	95°C	6 min	1 x
Melt curve analysis	55 - 95°C	10 sec	80 x (Δ0.5°C)

2.6.3 Data analysis

For the quantitative comparison of amplification rates of TNFα, the ΔΔCt method was used. The threshold cycle (Ct value) defines the fluorescence signal point where a background fluorescence is exceeded. For normalisation, the Ct value of the reference gene 18S was subtracted from the Ct value of the target gene (= ΔCt value). Relative gene expression of the target gene was then determined in relation to the untreated control (ΔΔCt) and is represented as '-fold change'.

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test using Graph Pad Prism (version 6.00, GraphPad Software Inc., San Diego, CA, USA. Data are expressed as mean ± SEM; n represents the number of different animals used.

2.6.4 Materials and equipment

Table 13: Specification of primers used for qPCR

	Sequence	Source
Rattus norvegicus 18S ribosomal RNA		
sense	5'-ACGATGCCGACTGGCGATGC-3'	Hoser *et al.* 2011
antisense	5'-GCTCCACCAACTAAGAACGGCCA-3'	Hoser *et al.* 2011
Rattus norvegicus tumor necrosis factor, mRNA		
sense	5'-TCAGCCTCTTCTCATTCCTG-3'	Michael *et al.* 2010
antisense	5'-GGCTACGGGCTTGTCACTCG-3'	Michael *et al.* 2010

Table 14: TNFα gene expression in ileum/jejunum preparations - Substances and consumables

Substance/consumable	**Supplier**
peqGOLD RNAPure	PEQLAB, Erlangen, Germany
water (nuclease-free)	Thermo Fisher Scientific Inc., Waltham, MA, USA
Oligo(dT)18 Primer	Thermo Fisher Scientific Inc., Waltham, MA, USA
dNTP Mix	Thermo Fisher Scientific Inc., Waltham, MA, USA
5x Reaction Buffer	Thermo Fisher Scientific Inc., Waltham, MA, USA
RiboLock™ RNase-Inhibitor	Thermo Fisher Scientific Inc., Waltham, MA, USA
RevertAid™ H Minus Reverse Transcriptase	Thermo Fisher Scientific Inc., Waltham, MA, USA
Reaction tubes (0.2, 0.5, 1.5 ml)	Sarstedt, Nürnbrecht, Germany
Transperent PCR foil, self-adhesive, DNase/RNase-free	Sarstedt, Nürnbrecht, Germany
96 well PCR-plates, DNase/RNase-free	PEQLAB, Erlangen, Germany

Table 15: TNFα gene expression in ileum/jejunum preparations - Equipment

Equipment	**Manufacturer**
Centrifuge ‘Biofuge Pico‘	Heraeus, Osterode, Germany
Spectrophotometer Ultrospec 2000	Pharmacia Biotech, Dübendorf, Germany
MJMini™ Personal Thermal Cycler	Bio-Rad, Munich, Germany
Plate centrifuge ‘Allegra’	Beckman Coulter, Krefeld, Germany
Realtime RT-PCR MyIQ™ Cycler	Bio-Rad, Munich, Germany

2.7 Cell culture

2.7.1 Differentiated THP-1 cells

THP-1 is a human leukemic cell line, originally derived from the peripheral blood of an one-year-old male infant with acute monocytic leukemia, which expresses distinct monocytic markers (Tsuchiya *et al.* 1980). In contrast to native human monocytes, this cell line offers the advantage of a homogeneous population, which markedly facilitates biochemical studies. Under the influence of phorbol esters such as TPA (12-O-tetradecanoylphorbol-13-acetate) or PMA (phorbol-12-myristate-13-acetate), THP-1 cells differentiate into macrophage-like cells (Tsuchiya *et al.* 1982), which are commonly used to model macrophage function.

Cell culture and differentiation of THP-1 cells

THP-1 cells (ATCC, TIB-202) in a concentration of 1-10 x 10^6 were cultured in RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) supplemented with fetal calf serum (FCS, 10 %; PAA Laboratories GmbH, Pasching, Austria) and penicillin/streptomycin (P/S, 1 %; Biochrom AG, Berlin, Germany) at standard cell culture conditions.

For the differentiation to macrophage-like cells, phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, Steinheim, Germany) in a concentration of 100 ng/ml was added to the culture medium and cells were allowed to differentiate for 48 hours at 37°C in 5 % CO_2 humidified air.

2.7.2 RAW 264.7 macrophages

The murine macrophage cell line RAW 264.7, which was established from murine tumours induced with Abelson leukemia virus by Raschke *et al.* in 1978, expresses properties of macrophages and serves as a suitable model for macrophage function (Raschke *et al.* 1978).

All experiments with this cell line were kindly allowed to be performed at the Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, in the laboratories of Prof. A. Hensel. In this context only aqueous (0.01 – 1000 µg/ml) and ethanolic myrrh extract (0.001 – 100 µg/ml) have been tested.

RAW 264.7 cells (ATCC, TIB-71) in a concentration of 1-10 x 10^6 were cultured in DMEM (high glucose, plus glutamine; PAA Laboratories GmbH, Pasching, Austria) supplemented with fetal calf serum (FCS, 10 %; Thermo Scientific, Waltham, USA), penicillin/streptomycin (P/S, 1 %; PAA Laboratories GmbH, Pasching, Austria) at standard cell culture conditions.

2.8 Determination of TNFα release (TNFα-ELISA)

2.8.1 LPS stimulation and substance incubation

After differentiation to macrophage-like cells, THP-1 cells were stimulated with LPS (100 ng/ml, Lipopolysaccharide from E. coli 0111:B4; Sigma-Aldrich, Steinheim, Germany) for four hours. LPS incubation polarises PMA treated THP-1 cells towards the pro-inflammatory M1-subtype and induces a strong pro-inflammatory response as it has been demonstrated before (Chanput *et al.* 2013).

To determine the influence of the plant extracts on inflammatory response e.g. TNFα release, the substances were incubated simultaneously with LPS (100 ng/ml) in their respective concentration. Budesonide (1 nM; Sigma-Aldrich, Steinheim, Germany) served as positive control. Medium or DMSO in applied concentrations served as vehicle control. For the determination of TNFα release, all plant extracts were tested in double determination on LPS-stimulated and untreated cells. All samples were incubated at standard cell culture conditions (37°C in 5 % CO_2 humidified air) for four hours.

After incubation, the supernatants were removed completely and after one centrifugation step the cell-free lysates were stored until further use at -80°C. The supernatants were used for determination of TNFα release and cytotoxicity testing (LDH-assay, see 2.10.2). Remaining cells on the cell culture plate were used for cell viability test (MTT-assay, see 2.10.1).

2.8.2 Test execution

For the determination of TNFα release by enzyme-linked immunosorbent assay (ELISA), the human TNF ELISA Kit II BD OptEIA™ (BD Biosciences, Franklin Lakes, NJ, USA) was used according to manufacturer's instructions.

Thus, after the high binding ELISA plate (96 well) has been coated with the antigen-specific capture antibody, the TNFα molecules in the collected supernatants were allowed to bind to the capture antibody. In a next step, a biotinylated detection antibody bound to the antigen providing a binding site for the enzyme, which catalysed a colour reaction, when the specific substrate (hydrogen peroxide and tetramethylbenzidin) was added. The formation of the coloured product, which correlates directly with the amount of TNFα in the samples, was detected spectrophotometrically at 450 nm with a reference wave length at 570 nm. For quantification, a standard curve was established for each experiment using known amounts of human TNFα (provided in kit).

2.8.3 Data analysis

Recording of the extinction data was done using ADAP basic software (Anthos Mikrosysteme GmbH, Krefeld, Germany).

Further data analysis was performed using Microsoft Excel (version 14.0, Microsoft cooperation, Redmond, WA, USA) and Graph Pad Prism (version 6.00, GraphPad Software Inc., San Diego, CA, USA) for statistical analysis.

TNFα release was expressed as % of the TNFα release induced by untreated or LPS-stimulated cells respectively. Data are expressed as mean ± SEM; n represents the number of independent experiments.

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test. For the determination of concentration effect relations, concentration-response curves and corresponding parameters such as EC/IC50 were obtained using non-linear regression by GraphPad Prism software. The selectivity index (SI) was determined using the IC50 value of cell viability and the IC50 value of TNFα release according to the following equation:

$$SI = \frac{IC_{50}\,A}{IC_{50}\,B}$$

SI: selectivity index
IC50 A: half maximal inhibitory concentration of cell viability (MTT assay)
IC50 B: half maximal inhibitory concentration of TNFα release

2.8.4 Materials and equipment

Table 16: TNFα release – Substances and consumables

Substance/consumable	Supplier
RPMI medium 1640 (1x), with glutamine	PAA Laboratories GmbH, Pasching, Austria
Fetal calf serum (FCS), heat inactivated	PAA Laboratories GmbH, Pasching, Austria
Penicillin 10.000 units/streptomycin 10 mg	Biochrom AG, Berlin, Germany
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany
Lipopolysaccharide from E. coli 055B5	Sigma-Aldrich, Steinheim, Germany
Budesonide	Sigma-Aldrich, Steinheim, Germany
Cell culture multiwell plates (96-well)	Techno Plastic Products AG, Trasadingen, Schweiz
ELISA-plates, Microlon, 96 well, high binding	Greiner Bio-One GmbH, Frickenhausen, Germany
Neubauer counting chamber	Fein-Optik, Bad Blankenburg, Germany
Serological pipettes	Sarstedt AG & Co., Nümbrecht, Germany
Cell culture flasks (75 cm^2)	Sarstedt AG & Co., Nümbrecht, Germany
Centrifuge tubes (15 ml, 50 ml)	Techno Plastic Products AG, Trasadingen, Schweiz
Human TNF ELISA Kit II BD OptEIA™	BD Biosciences, Franklin Lakes, NJ, USA

Table 17: TNFα release – Equipment

Equipment	Manufacturer
Laminar Flow Bench Biowizard Kojair®	UniEquip, Leipzig, Germany
Multi-gas incubator	Sanyo Sales & Marketing Europe GmbH, Munich, Germany
Freezer	Harris Manufacturing Company, North Billerica, M.A.
UV/VIS-spectrometer Anthos Reader 2010	Anthos Mikrosysteme GmbH, Krefeld, Germany
Test tube shaker lab dancer	VWR International GmbH, Darmstadt, Germany
Vortex shaker Reax 2000	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Centrifuge HERMLE Z232K	UniEquip, Leipzig, Germany

2.9 Determination of nitric oxide release

2.9.1 LPS stimulation and substance incubation

To induce an inflammatory response, cells were stimulated with LPS (100 ng/ml, Lipopolysaccharide from E. coli 0111:B4; Sigma-Aldrich, Steinheim, Germany) for 24 hours.

To determine the influence of the plant extracts on the inflammatory response represented by nitric oxide (NO) release, the substances were incubated simultaneously with LPS in their respective concentration. The inducible nitric oxide synthase inhibitor aminoguanidine (100 µg/ml; Caymann Chemical Company, Ann Arbor, USA) served as positive control. Medium or DMSO in applied concentrations served as vehicle control. For the determination of NO release, all plant extracts were tested in triple determination on LPS-stimulated and untreated cells. All samples were incubated at standard cell culture conditions (37°C in 5 % CO_2 humidified air) for 24 hours.

After incubation, the supernatants were removed completely and used for determination of NO release. Remaining cells on the cell culture plate were used for cell viability test (MTT assay, see 2.10.1).

2.9.2 Test execution

After LPS stimulation, nitric oxide (NO) production was determined using Griess reagent. In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. After addition of Griess reagent (1% sulfanilamide; 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride; 4.3 % phosphoric acid), the resulting nitrite in the samples can be detected spectrophotometrically after the induced diazonium coupling reaction.

Thus, after LPS and substance incubation the collected supernatant was incubated with Griess reagent. After an incubation time of 30 minutes, the formation of the azo compound was measured spectrophotometrically at 540 nm with a reference wave length at 690 nm.

2.9.3 Data analysis

Recording of the extinction data was done using Sunrise microplate reader software (Tecan, Grödig, Austria). Further data analysis was performed using Microsoft Excel (version 14.0, Microsoft cooperation, Redmond, WA, USA) and Graph Pad Prism (version 6.00, GraphPad Software Inc., San Diego, CA, USA) for statistical analysis.

Nitric oxide release was expressed as % of the nitric oxide release induced by untreated or LPS-stimulated cells respectively. Data are expressed as mean ± SEM; n represents the number of independent experiments.

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test.

2.9.4 Materials and equipment

Table 18: Nitric oxide release – Substances and consumables

Substance/consumable	Supplier
DMEM high glucose, incl. glutamin	PAA Laboratories GmbH, Pasching, Austria
Fetal calf serum (FCS), heat inactivated	Thermo Scientific, Waltham, USA
Penicillin 10.000 units/streptomycin 10 mg	PAA Laboratories GmbH, Pasching, Austria
Griess reagent for nitrite	Fluka, Buchs, Switzerland
Lipopolysaccharide from E. coli 055:B5	Sigma-Aldrich, Steinheim, Germany
Aminoguanidine hydrochloride	Cayman Chemical, Ann Arbor, USA
Microtiter multiwell plates, 96 well	Sarstedt AG & Co., Nümbrecht, Germany
Serological pipettes	Sarstedt AG & Co., Nümbrecht, Germany

Table 19: Nitric oxide release – Equipment

Equipment	Manufacturer
Laminar Flow System Biowizard	Kojair, Vilppula, Finland
Binder CO_2 Incubator CB150	WTB Binder, Tuttlingen, Germany
Sunrise microplate reader	Tecan, Grödig, Austria

2.10 Cytotoxicity testing

2.10.1 MTT-assay

The MTT assay determines the viability of cells by mitochondrial reduction of yellow soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) to purple formazan in metabolically active cells. Briefly, after macrophages or macrophage-like cells were stimulated with LPS, the supernatant has been removed and remaining cells were treated with 100 µl MTT (0.3 mg/ml in PBS) per well for two hours. Triton X was used as control for complete cell death. After the incubation period, cells were lysed by addition of 100 µl lysis buffer. After complete cell lysis, the amount of resulting purple formazan was detected spectrophotometrically at 570 nm.

2.10.2 LDH-assay

The LDH-assay is a colorimetric test to determine cell membrane integrity as an indicator of cell death. It is based on the reduction of nicotinamide adenine dinucleotide (NAD+) by cytosolic lactate dehydrogenase (LDH) which is released when membrane integrity is impaired. The resulting NADH is utilised by the enzyme diaphorase in the stoichiometric conversion of a yellow tetrazolium dye into a red formazan product, which can be detected spectrophotometrically.

Thus, after differentiation to macrophage-like cells and stimulation with LPS, the collected cell free supernatant was incubated with the substrate solution (diaphorase/NAD). Triton X was used as control for complete cell death. After an incubation time of 30 minutes the enzymatic reaction was stopped by addition of hydrochloric acid (HCl, 1 N) and the formation of the red formazan was measured spectrophotometrically at 492 nm with a reference wave length at 620 nm.

2.10.3 Data analysis

Recording of the extinction data was done using ADAP basic software (Anthos Mikrosysteme GmbH, Krefeld, Germany).

Further data analysis was performed using Microsoft Excel (version 14.0, Microsoft cooperation, Redmond, WA, USA) and Graph Pad Prism (version 6.00, GraphPad Software Inc., San Diego, CA, USA) for statistical analysis.

Metabolic activity and LDH-release were expressed as % of the metabolic activity or LDH-release of untreated cells respectively. Data are expressed as mean ± SEM; n represents the number of independent experiments.

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test.

2.10.4 Materials and equipment

Table 20: Cytotoxicity testing - Substances and consumables

Substance/consumable	Supplier
Hydrochloric acid (1 N HCl)	Grüssing GmbH, Filsum, Germany
Triton-X 100	Sigma-Aldrich, Steinheim, Germany
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich, Steinheim, Germany
Cytotoxicity Detection Kit (LDH)	Roche Diagnostics GmbH, Mannheim, Germany
Serological pipettes	Sarstedt AG & Co., Nümbrecht, Germany

Table 21: Cytotoxicity testing - Substances and consumables – lysis buffer

Substance	Amount	Supplier
Lysis buffer		
Sodium dodecyl sulfate (SDS)	50.0 g	Sigma-Aldrich, Steinheim, Germany
Dimethylformamide (DMF)	80 ml	Grüssing GmbH, Filsum, Germany
Aqua purificata	ad 250 ml	

Table 22: Cytotoxicity testing - Equipment

Equipment	Manufacturer
Laminar Flow Bench Biowizard Kojair®	UniEquip, Leipzig, Germany
Multi-gas incubator	Sanyo Sales & Marketing Europe GmbH, Munich, Germany
UV/VIS-spectrometer Anthos Reader 2010	Anthos Mikrosysteme GmbH, Krefeld, Germany
Vortex shaker Reax 2000	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany

2.11 Native human M1-macrophages

2.11.1 Preparation of human monocytes and macrophage differentiation

Whole blood was obtained from healthy volunteers after informed consent. Blood donation was performed at the Institute for Transfusion Medicine (Blood donation centre, Institute for Transfusion Medicine, Leipzig University Hospital).

Monocytes were isolated using density gradient centrifugation. Thus, 25 ml whole blood (treated with citrate phosphate dextrose solution as anticoagulant) were layered on 20 ml Ficoll separating solution with a specific density of 1.077 g/ml (Biochrom AG, Berlin, Germany) and separated by centrifugation at 400x g (without break) for 20 minutes at room temperature. The interphase, containing peripheral blood mononuclear cells (PBMCs) like lymphocytes, monocytes and thrombocytes was carefully removed and washed twice with PBS. An additional centrifugation step at 100x g for 15 minutes with PBS was performed to separate thrombocytes which remain in the supernatant. A scheme illustrating the PBMC isolation process is presented in Figure 10.

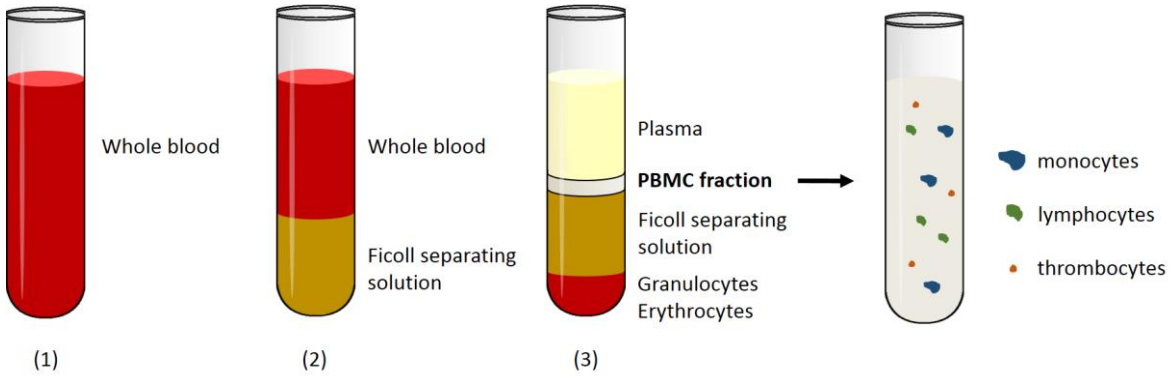

Figure 10: Isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation (adapted from GE Healthcare)
(1) Whole blood was obtained from healthy volunteers after informed consent.
(2) Ficoll separation solution with a specific density of 1.077 g/ml was layered under the whole blood fraction.
(3) After density gradient centrifugation (400x g; 20 minutes; without break) the peripheral blood mononuclear cell (PBMC) fraction was separated and collected.

For the isolation of monocytes from the PBMC fraction, Monocytes isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), an indirect labelling system, was used according to manufacturer's instructions. In a first step, unspecific binding sites on the cell surface were blocked using blocking reagents. Non-monocytes, i.e. T cells, B cells, dendritic cells and basophils were afterwards indirectly magnetically labelled using a mixture of biotin-conjugated antibodies (primary labelling: anti-CD3, anti-CD7, anti-CD16, anti-CD19, anti-CD56, anti-CD123 and glycophorin A) and anti-biotin microbeads (secondary labelling).

Using magnetic separation columns (LS MACS Columns, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), labelled non-monocytic cells were retained, while unlabelled monocytes were eluted and collected.

Quality control for purity of isolated monocytes

Purity of the enriched monocyte fraction was evaluated by flow cytometry using fluorochrome-conjugated CD14-antibodies (Anti-Human CD14 PE; eBioscience, Frankfurt, Germany). Thus, 5 x 10^5 cells were suspended in 50 µl PBS containing bovine serum albumin (BSA, 1 %; Sigma Aldrich, Steinheim, Germany) and 5 µl antibody solution as well as isotype control (Mouse IgG1 K Isotype Control PE; eBioscience, Frankfurt, Germany) was added and incubated for 30 minutes in the dark. After two washing steps, cells were suspended in phosphate buffered saline (PBS; Amresco, Solon, OH, USA) and analysed by flow cytometry (BD FACSCalibur™, BD Bioscience, Heidelberg, Germany).

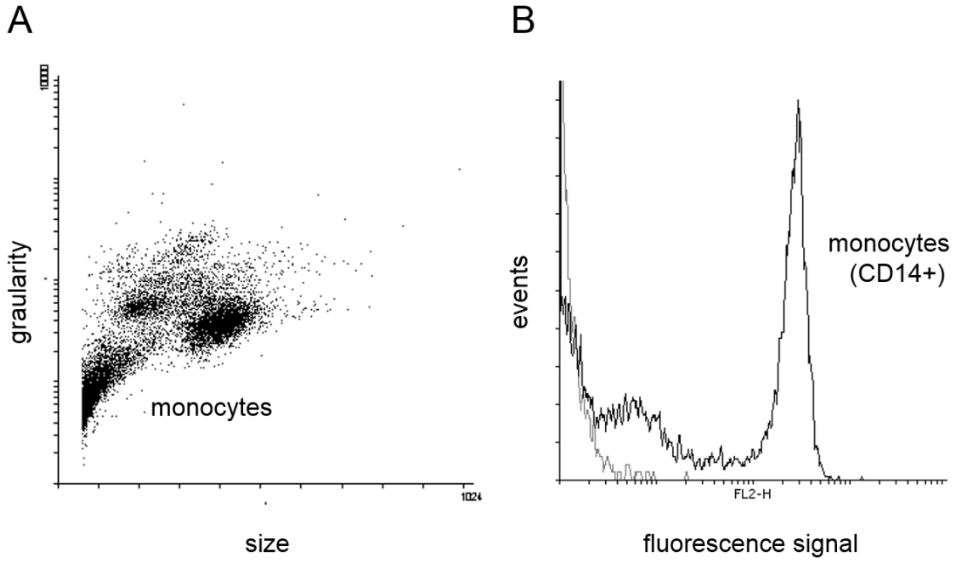

Figure 11: Detection of CD14 in isolated human monocytes.
Cells were incubated with anti-CD14 for 30 minutes and analysed using flow cytometry. [A] shows size and granularity of isolated monocytes. Fluorescence intensity of 10^4 cells was measured at 530 nm. [B] 80.94 % (± 6.7; n = 3) of the detected cell were CD14-positive monocytes.

Differentiation to M1-macrophages

Isolated monocytes were counted using a Neubauer improved counting chamber, adjusted to 1 x 10^6 cells/ml in RPMI 1640 supplemented with fetal calf serum (FCS, 10 %; Sigma-Aldrich, Steinheim, Germany), penicillin/streptomycin (P/S, 1 %; Sigma-Aldrich, Steinheim, Germany) and cultured at standard cell culture conditions.

For the differentiation to macrophages, granulocyte-macrophage colony-stimulating factor (GM-CSF; Life Technologies, Carlsbad, CA, USA) in a concentration of 100 ng/ml was added to the culture medium.

Monocytes were allowed to differentiate for 6 days at 37°C in 5 % CO_2 humidified air, whereby a complete medium change was performed at day 2 and 4.

2.11.2 LPS/IFNγ stimulation and incubation of plant extracts

After differentiation to macrophages, the isolated cells were stimulated with a mixture of 100 ng/ml LPS (lipopolysaccharide from E. coli 0111:B4; Sigma-Aldrich, Steinheim, Germany) and 10 ng/ml interferon-γ (IFNγ, Biomol, Hamburg, Germany) for 24 hours to polarise macrophages towards the pro-inflammatory M1-subtype as it has been demonstrated by Jaguin *et al.* and to induce an inflammatory response (Jaguin *et al.* 2013).

In order to determine the influence of the plant extracts on the inflammatory response, the substances were incubated simultaneously with the LPS/IFNγ mixture in their respective concentration. Based on previous cell culture experiments, optimal incubation conditions and concentrations were determined. Subsequently, ethanolic myrrh extract (30 µg/ml), aqueous myrrh extract (500 µg/ml), chamomile flower extract (200 µg/ml) and coffee charcoal extract (500 µg/ml) were tested. All samples were incubated at standard cell culture conditions (37°C in 5 % CO_2 humidified air) for 24 hours.

2.11.3 RNA isolation

After LPS/IFNγ stimulation and substance incubation, total RNA was isolated from adherent macrophages by column separation using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Thus, after cell lysis with lysis buffer, lysates were collected and transferred to the separation columns. During centrifugation of the columns (8000x g; 15 s), total RNA bound to the column membrane. After three washing steps, residual liquid was removed by centrifugation for 2 minutes at 9000x g. For elution of the total RNA, 30 µl RNase free water was added to the column and incubated for 2 minutes. In a final centrifugation step (8000x g; 1 min), RNA was eluted from the column membrane, quickly frozen and stored at -80°C until further use.

Before further processing, RNA quality and integrity was determined electrophoretically at the Core Unit DNA Technologies of the Interdisciplinary Centre for Clinical Research (IZKF, Medical Faculty, University of Leipzig) using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Thus, RNA agarose-gel electrophoresis was performed to separate RNA by size. Subsequently, the RNA integrity number (RIN) was determined (Figure 4). The RIN allows for classification of total RNA, based on a numbering

system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. Based on this assessment the quality of most of the RNA samples was found sufficient for gene expression analysis. However, the quality of isolated RNA from macrophages treated with aqueous myrrh extract (sample-ID: MYA500) was found reduced with a RIN value of 3.10. This could be related to the fact, that RNA yield was comparably low after isolation as well. In this case, centrifugal vacuum evaporation was performed to concentrate the RNA sample before gene expression analysis.

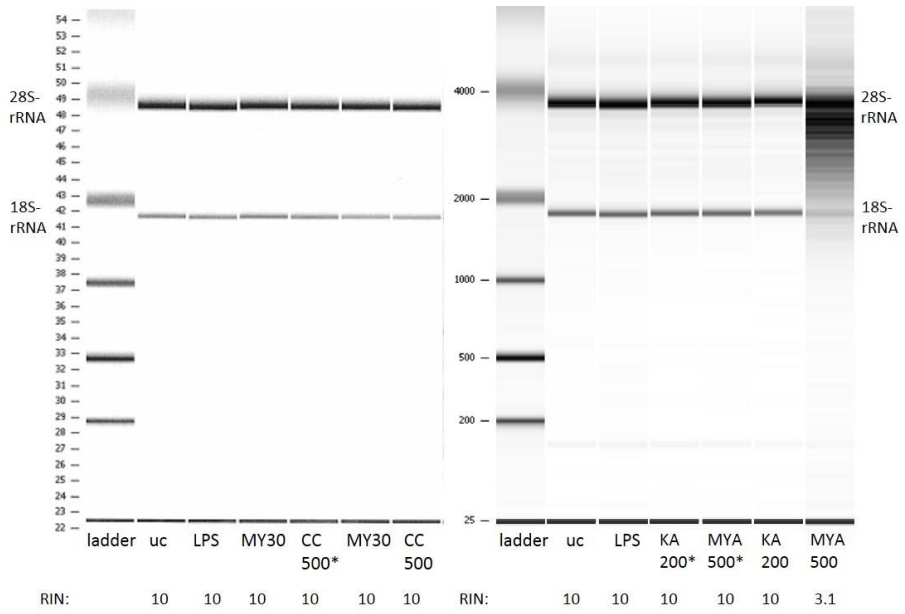

Figure 12: Quality assessment of total RNA by agarose-gel electrophoresis.
Electropherogram and RNA Integrity Number (RIN) of mRNA samples from human macrophages incubated with LPS/IFNγ (LPS) and ethanolic myrrh extract (MY30*), coffee charcoal extract (CC500*), chamomile flower extract (KA200*) or aqueous myrrh extract (MYA500*) or single incubation with ethanolic myrrh extract (MY30), coffee charcoal (CC500), chamomile flower extract (KA200) or aqueous myrrh extract (MYA500) for 24 hours. All samples show specific lines for 28S- and 18S-ribosomal RNA.

2.11.4 cDNA synthesis

Synthesis of cDNA was performed at the Core Unit DNA Technologies of the Interdisciplinary Centre for Clinical Research (IZKF, Medical Faculty, University of Leipzig). Briefly, poly(A) RNA (mRNA) was amplified in a total RNA sample using MessageBOOSTER™ cDNA Synthesis Kit for qPCR (Epicentre Technologies Corporation, Madison, WI, USA). Using this method, the poly(A) RNA component of a total RNA sample is reverse transcribed into first-strand cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California, USA). Next, the RNA component of the cDNA:RNA hybrid is digested into small RNA fragments by RNase H and the second strand cDNA is synthesised. During this *in vitro* transcription the double stranded cDNA is transcribed and high yields of antisense-RNA (aRNA) are obtained. In a

clean-up process the aRNA is purified by spin column chromatography using RNeasy MiniElute Cleanup (Qiagen, Hilden, Germany). Finally, the purified aRNA is reverse transcribed into first-strand cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California, USA), quickly frozen and stored at -80°C until further use.

2.11.5 Microarray gene expression analysis

Gene expression analysis was performed for all plant extracts in double determination at the Core Unit DNA Technologies of the Interdisciplinary Centre for Clinical Research (IZKF, Medical Faculty, University of Leipzig) using a HumanHT-12 v4 Expression BeadChip (Illumina Inc., San Diego, CA, USA). In the first gene expression analysis procedure (20/03/2014), ethanolic myrrh extract (30 µg/ml) and coffee charcoal extract (500 mg/ml) were tested. Chamomile flower extract (200 µg/ml) and aqueous myrrh extract (500 µg/ml) were tested in a second gene expression analysis procedure (17/04/2014).

2.11.6 Data analysis

Results of microarray gene expression analysis was provided as text-file including information such as the distinct gene ID (Probe_ID), gene symbol, and the corresponding signal values. Processing of raw data was performed using Microsoft Excel (version 14.0, Microsoft cooperation, Redmond, WA, USA). Thus, double determined values were averaged and values below 4.0 were set 1.0 for correction of background noise. For the identification of genes that were affected by the particular treatments, ratios were determined to detect changes relative to untreated cells or LPS/IFNγ-treated cells.

Classification of affected genes was performed using the software based gene ontological enrichment analysis ‘WEB-based GEne SeT AnaLysis Toolkit’ (WebGestalt software (version 1/30/2013) provided by the Vanderbilt University. Genes that were up- or down-regulated by factor ≥ 10 were entered into the platform using their Reference Sequence (RefSeq)-ID. A complete list of all analysed genes was used as reference data set. Statistical analysis was performed using hypergeometric test followed by Multiple Test Adjustment according to Benjamini-Hochberg (BH) which reported an adjusted p-value (adjP). Results were clustered and mapped on the base of the “Gene Ontology”. The Gene Ontology allows the annotation of homologous gene and protein sequences in multiple organisms using a common vocabulary for the description of the biological process, molecular function and cellular component of genes (Ashburner *et al.* 2000).

Gene ontological (GO) categories with an adjusted p-value ≤ 0.05 were considered significantly enriched. If more than ten categories were significantly enriched, only categories with the ten lowest significance values were highlighted to allow better visualisation.

2.11.7 Methods and equipment

Table 23: Native human M1-macrophages - Substances and consumables

Substance/consumable	Supplier
Ficoll separating solution	Biochrom AG, Berlin, Germany
Anti-Human CD14-PE	eBioscience, San Diego, CA, USA
Phosphate Buffered Saline, PBS (1x)	Amresco, Solon, OH, USA
Albumin from bovine serum	Sigma Aldrich, Steinheim, Germany
Mouse IgG1 K Isotype Control PE	eBioscience, Frankfurt, Germany
RPMI medium 1640 (1x), with glutamine	Gibco, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA
Newborn calf serum (FCS), heat inactivated	Sigma Aldrich, Steinheim, Germany
Penicillin 10.000 units/streptomycin 10 mg	Sigma Aldrich, Steinheim, Germany
Recombinant GM-CSF	Life Technologies, Carlsbad, CA, USA
Lipopolysaccharide from E. coli 0111:B4	Sigma-Aldrich, Steinheim, Germany
Interferon gamma, human recombinant	Biomol, Hamburg, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
Anti-Human CD80-PE	eBioscience, San Diego, CA, USA
Anti-Human CD284-PE	eBioscience, San Diego, CA, USA
Anti-Human CD163-PE	eBioscience, San Diego, CA, USA
Monocytes isolation Kit II	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
LS MACS Columns	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Agilent RNA 6000 Nano Kit	Agilent Technologies, Inc., Santa Clara, CA, USA
NunclonTM Surface, 6 wells	Nunc, Langenselbold, Germany
Serological pipettes	Sarstedt AG & Co., Nümbrecht, Germany

Table 24: Native human M1-macrophages - Equipment

Equipment	**Manufacturer**
Centrifuge ‘Laborfuge 400R’	Heraeus Holding GmbH, Hanau, Germany
BD FACSCalibur	BD Bioscience, Heidelberg, Germany
Agilent 2100 Bioanalyzer	Agilent Technologies, Inc., Santa Clara, CA, USA
Vacuum Concentrator, Uniequip Univapo 100 H	UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg, Germany
Illumina HighScan Array Scanner	Illumina, San Diego, CA, USA

3 Results

3.1 Spasmolytic activity of Myrrhinil-Intest® components

3.1.1 Spasmolytic activity of chamomile flower extract

For investigation of spasmolytic activity, isometric contractions were measured using an organ bath equipment. They were induced by acetylcholine (ACh) applied directly into the organ baths with a final bath concentration of 1 mM.

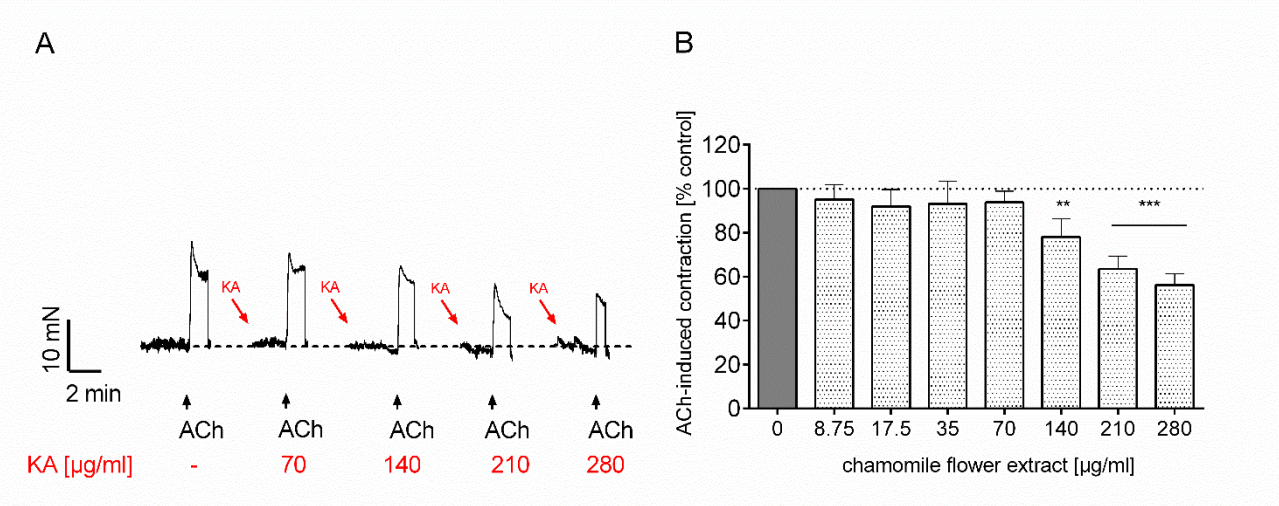

Figure 13: Influence of chamomile flower extract (KA) on acetylcholine (ACh)-induced contractions in untreated ileum/jejunum preparations (A: representative original recording; B: statistical analysis).
ACh (1 mM)-induced contractions were determined using isometric tension measurement. Chamomile flower extract (KA, 8.75 – 280 µg/ml) was applied two minutes prior induction of contraction. ACh-induced contractions were determined using isometric tension measurement. Data are presented as mean ± SEM; the vertical line in (A) represents basal tone; ** $p<0.01$ vs. control, *** $p<0.001$ vs. control; n = 5-10.

Application of chamomile flower extract (KA, 8.75 – 280 µg/ml) into the organ bath two minutes prior induction of contraction resulted in a concentration-dependent decrease of ACh-induced contraction down to 56 % ± 5.2 (% control) (Figure 13; 140 µg/ml KA: 78.06±8.2, $p<0.01$; 210 µg/ml KA: 63.48±5.9, $p<0.001$; 280 µg/ml KA: 56.11±5.2, $p<0.001$; in % control).

To investigate spasmolytic activity in inflamed tissue, inflammation was induced *in vitro* and ACh-induced contractions were assessed (Figure 14).

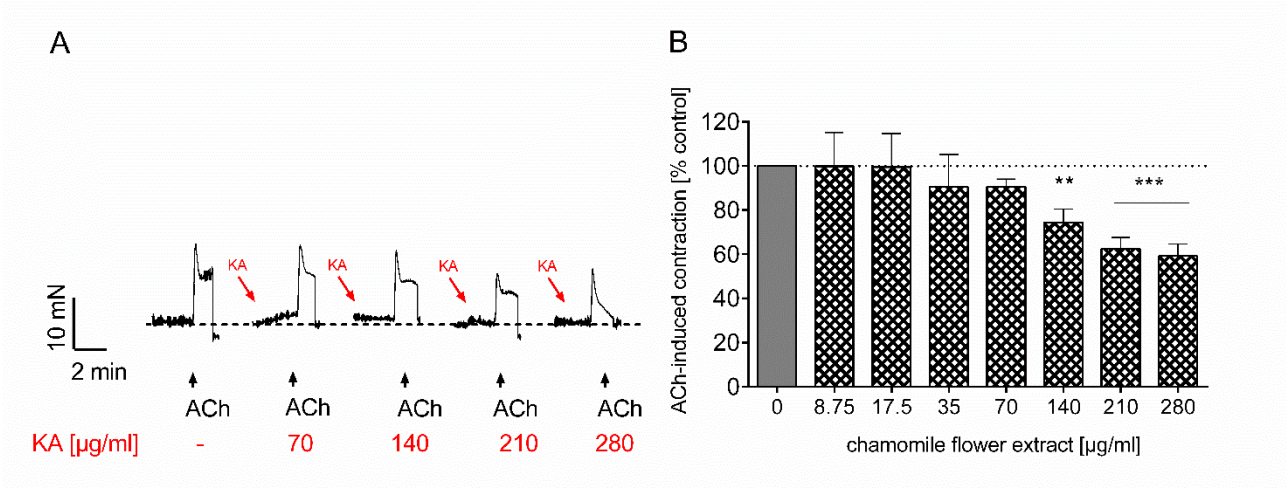

Figure 14: Influence of chamomile flower extract (KA) on acetylcholine (ACh)-induced contractions in inflamed ileum/jejunum preparations (A: representative original recording; B: statistical analysis).
ACh (1 mM)-induced contractions were determined using isometric tension measurement. Chamomile flower extract (KA, 8.75 – 280 µg/ml) was applied two minutes prior induction of contraction. Data are presented as mean ± SEM; the vertical line in (A) represents basal tone; ** $p<0.01$ vs. control, *** $p<0.001$ vs. control; n = 5-10.

Application of chamomile flower extract (KA, 8.75 – 280 µg/ml) resulted in a concentration-dependent decrease of ACh-induced contraction down to 59 % ± 5.2 (% control) (Figure 14; 140 µg/ml KA: 74.4±6.0, $p<0.01$; 210 µg/ml KA: 62.54±5.0, $p<0.001$; 280 µg/ml KA: 59.23±5.4, $p<0.001$; in % control).

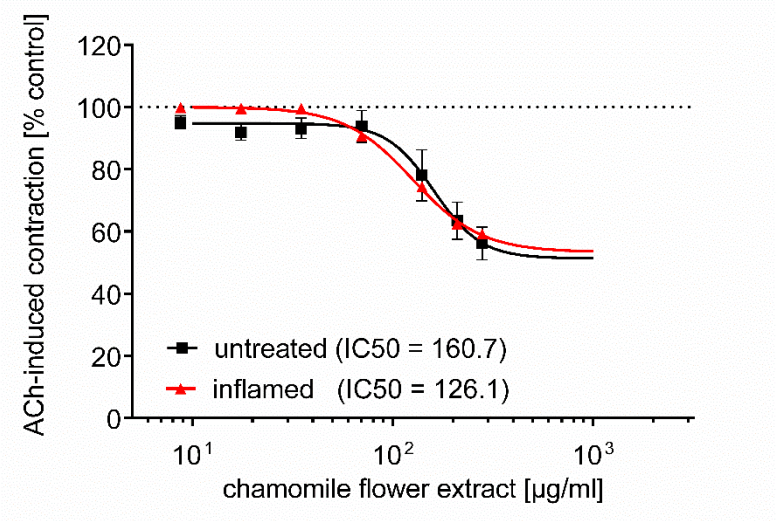

Figure 15: Concentration-response curves in untreated and inflamed ileum/jejunum preparations.
Effect of chamomile flower extract (8.75 – 280 µg/ml) on ACh-induced contraction in inflamed and untreated ileum/jejunum preparations. Data are presented as mean ± SEM and non-linear regression curve for untreated (■) and inflamed (▲) preparations (n = 5-10).

Comparing the concentration-response curves of the chamomile flower extract influence on ACh-induced contractions between untreated and inflamed preparations, no significant difference was found with regard to the half maximal inhibitory concentration (Figure 15; IC50 – untreated preparations: 160.7 µg/ml; IC50 – inflamed preparations: 126.1 µg/ml; Comparison of Fits [IC50]: p=0.41).

3.1.2 Spasmolytic activity of ethanolic myrrh extract

Effects on acetylcholine-induced contraction

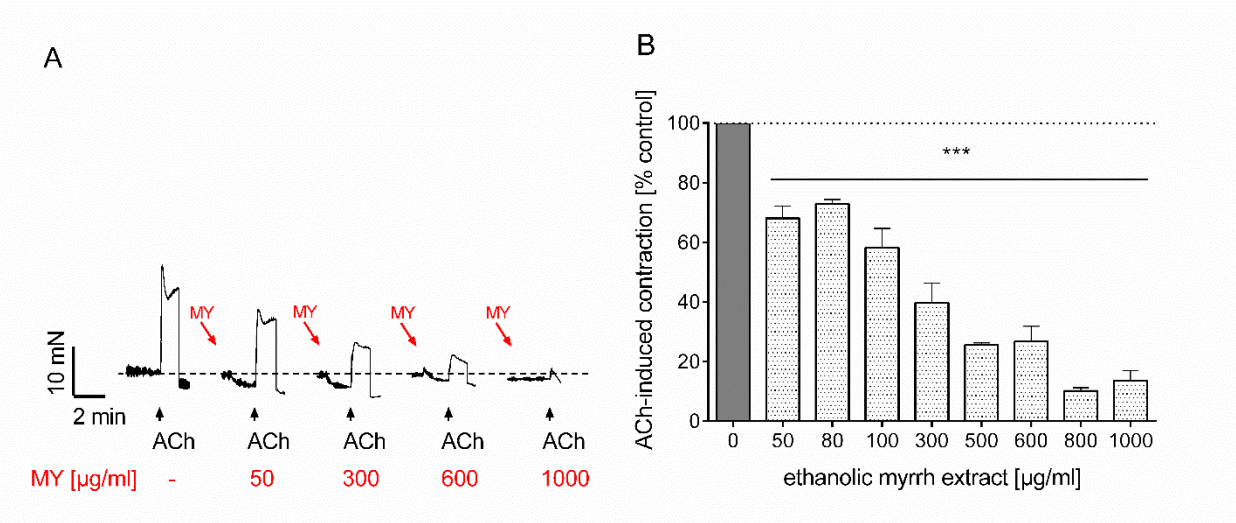

Figure 16: Influence of ethanolic myrrh extract (MY) on acetylcholine (ACh)-induced contractions in untreated ileum/jejunum preparations (A: representative original recording; B: statistical analysis).
ACh (1 mM)-induced contractions were determined using isometric tension measurement. Ethanolic myrrh extract (50 – 1000 µg/ml) was applied two minutes prior induction of contraction. Data are presented as mean ± SEM; the vertical line in (A) represents basal tone; *** $p<0.001$ vs. control; n = 3.

Application of ethanolic myrrh extract (50 – 1000 µg/ml) into the organ bath two minutes prior induction of contraction resulted in a concentration-dependent decrease of ACh-induced contraction down to 10 % ± 5.2 (% control) (Figure 16; 50 µg/ml MY: 68.13±4.1; 80 µg/ml MY: 72.95±1.5; 100 µg/ml MY: 58.23±6.4; 300 µg/ml MY: 39.70±6.4; 500 µg/ml MY: 25.60±0.67; 600 µg/ml MY: 26.70±5.1; 800 µg/ml MY: 10.10±1.1; 1000 µg/ml MY: 13.57±3.4; $p<0.001$; in % control).

To investigate spasmolytic activity in inflamed tissue, inflammation was induced *in vitro* and ACh-induced contractions were assessed (Figure 17).

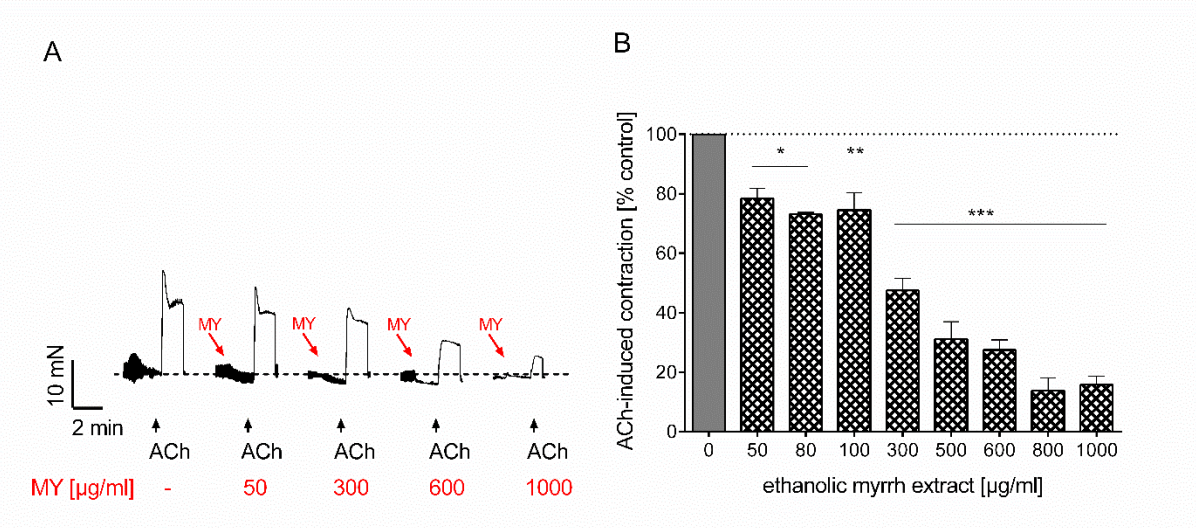

Figure 17: Influence of ethanolic myrrh extract (MY) on acetylcholine (ACh)-induced contractions in inflamed ileum/jejunum preparations (A: representative original recording; B: statistical analysis).
ACh (1 mM)-induced contractions were determined using isometric tension measurement. Ethanolic myrrh extract (50 – 1000 µg/ml) was applied two minutes prior induction of contraction. Data are presented as mean ± SEM; the vertical line in (A) represents basal tone (); * $p<0.05$ vs. control, ** $p<0.01$ vs. control, *** $p<0.001$ vs. control; n = 3.

Application of ethanolic myrrh extract (50 – 1000 µg/ml) to the organ bath two minutes prior induction of contraction resulted in a concentration-dependent decrease of ACh-induced contraction down to 14 % ± 4.3 (% control) (Figure 17; 50 µg/ml MY: 78.48±3.3; 80 µg/ml MY: 73.10±0.6; 100 µg/ml MY: 74.50±5.8; 300 µg/ml MY: 47.50±4.1; 500 µg/ml MY: 31.16±8.8; 600 µg/ml MY: 27.45±3.5; 800 µg/ml MY: 13.9±4.3; 1000 µg/ml MY: 15.82±2.8; $p<0.001$; in % control).

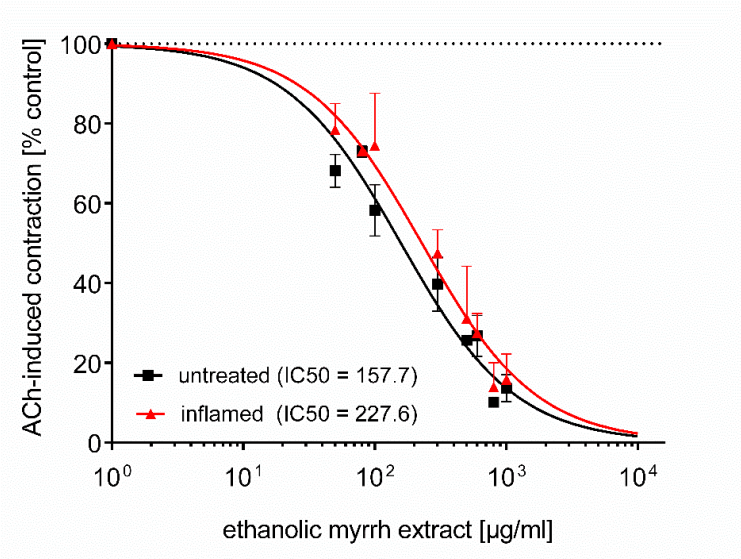

Figure 18: Concentration-response curves in untreated and inflamed ileum/jejunum preparations.
Effect of ethanolic myrrh extract (50 – 1000 µg/ml) on ACh-induced contraction in inflamed and untreated ileum/jejunum preparations. Data are presented as mean ± SEM and non-linear regression curve for untreated (■) and inflamed (▲) preparations (n = 6).

Comparing the concentration-response curves of the ethanolic myrrh extract influence on ACh-induced contractions between untreated and inflamed preparations, higher concentrations were necessary in inflamed tissue to induce half maximal inhibition. (Figure 18; IC50 – untreated preparations: 157.7 µg/ml; IC50 – inflamed preparations: 227.6 µg/ml; Comparison of Fits [IC50]: p=0.0047).

Effects on basal muscular tone

To determine the effect of ethanolic myrrh extract on basal muscular tone, isometric tension measurement was performed and muscular tone was recorded.

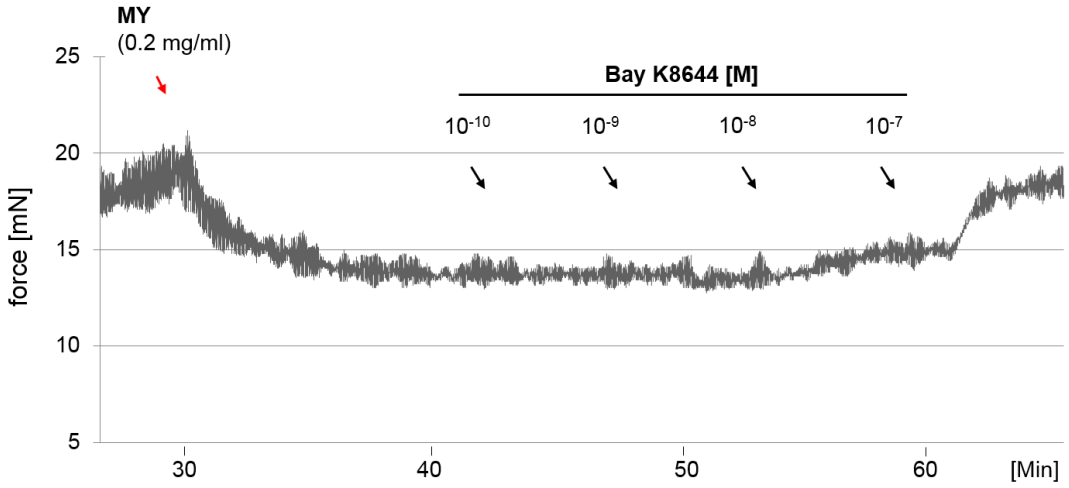

Figure 19: Effect of ethanolic myrrh extract (MY; 0.2 mg/ml) on basal muscular tone of untreated rat ileum/jejunum preparations.
Isometric tension measurement was performed with isolated rat ileum/jejunum preparations (representative original recording). Application of ethanolic myrrh extract (MY; 0.2 mg/ml) at minute 30 directly to the organ baths decreased basal muscular tone. Subsequent cumulative administration of L-type calcium channel agonist Bay K8644 (10^{-10} – 10^{-7} M) reversed the decrease in muscular tone.

Application of ethanolic myrrh extract in a concentration of 0.2 mg/ml directly into the organ baths resulted in a decrease in muscle tone (Figure 19). After cumulative application of the calcium channel agonist Bay K8644 (10^{-10} – 10^{-7} M), the effect could be antagonised and the muscle tone was restored to its initial level.

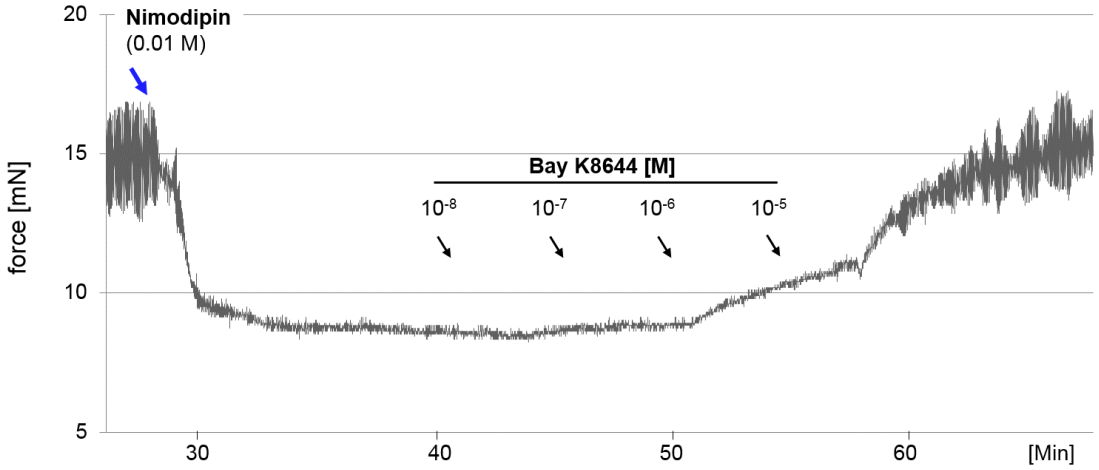

Figure 20: Effect of nimodipine (0.01 M) on basal muscular tone of untreated rat ileum/jejunum preparations.
Isometric tension measurement was performed with isolated rat ileum/jejunum preparations (representative original recording). Application of nimodipine at minute 30 directly to the organ baths decreased basal muscular tone. Subsequent cumulative administration of L-type calcium channel agonist Bay K8644 (10^{-8} – 10^{-5} M) reversed the decrease in muscular tone.

Application of the well-known calcium channel antagonist nimodipine (0.01 M) led likewise to an inhibition of basal muscular tone (Figure 20). After cumulative application of the calcium channel agonist Bay K8644 (10^{-8} – 10^{-5} M), the effect was antagonised and the muscle tone was restored to its initial level.

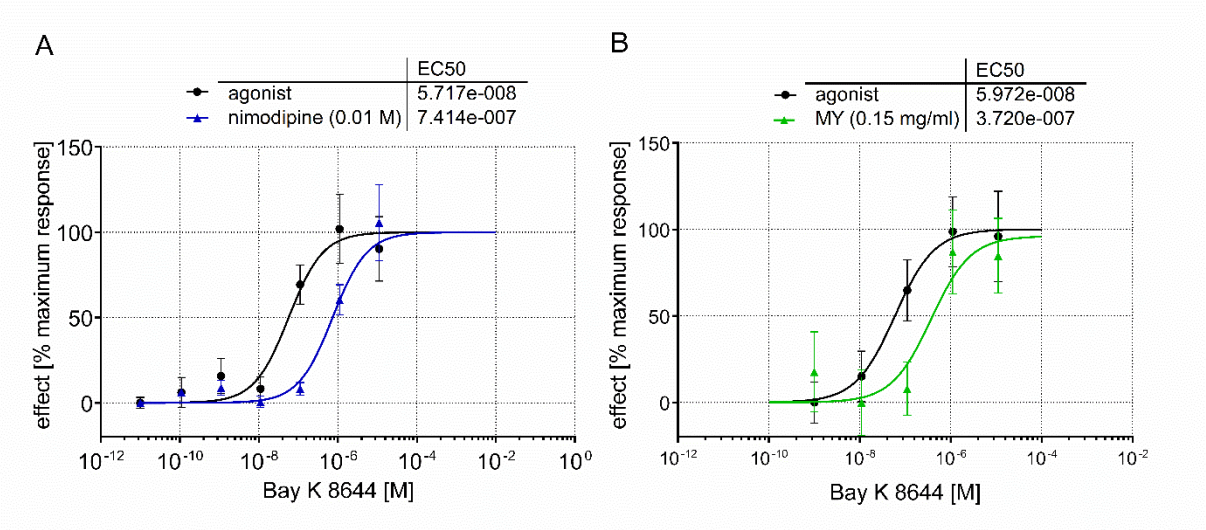

Figure 21: Bay K8644 concentration-response curves with and without antagonist (A: nimodipine, B: ethanolic myrrh extract)
Effect of pre-treatment with nimodipine (A, 0.01 M) and ethanolic myrrh extract (B, MY 0.15 mg/ml) on the agonistic (Bay K8644, 10^{-11} – 10^{-5} M) concentration-response curve measuring basal muscular tone of rat ileum/jejunum preparations. Data are presented as mean ± SEM and non-linear regression curve with and without pre-treatment of nimodipine and MY respectively, n = 8-10.

Concentration-response curves of Bay K8644 with and without pre-treatment of nimodipine (A) and ethanolic myrrh extract (B) are presented in Figure 21. Ethanolic myrrh extract in a concentration of 0.15 mg/ml led to a significant EC50 shift from 5.9E-8 M to 3.7E-7 M (Comparison of Fits [IC50; Emax]: p=0.0008). In comparison pre-treatment with nimodipine (0.01 mM) resulted in an EC50 shift from 5.7E-8 M to 7.4E-7 M (Comparison of Fits [IC50; Emax]: p<0.0001). The maximum effect of the agonist Bay K8644 was not significantly altered in this concentrations.

Additionally, increasing concentrations of ethanolic myrrh extract were tested to get a more precise picture of the antagonistic properties (Figure 22). Furthermore, these experiments were carried out using untreated (A) and inflamed (B) intestinal preparations.

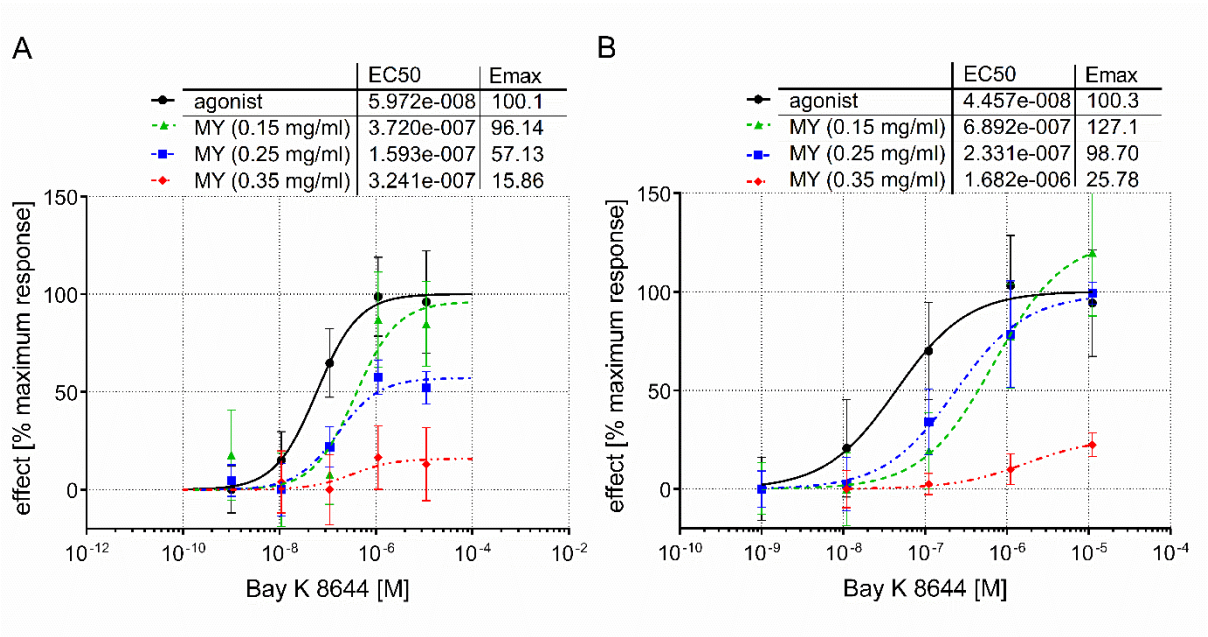

Figure 22: Bay K8644 concentration-response curves after pre-treatment with increasing concentrations of ethanolic myrrh extract.
Effect of pre-treatment with ethanolic myrrh extract (0.15 – 0.35 mg/ml) on the agonistic (Bay K8644, 10^{-9} - 10^{-6} M) concentration-response curve measuring basal muscular tone of untreated (A) and inflamed (B) rat ileum/jejunum preparations. Data are presented as mean ± SEM and non-linear regression curves; the table shows maximum effect (Emax) and EC50-values of the agonistic concentration-response relation dependent on pre-treatment with increasing myrrh extract concentrations (n = 4).

Pre-treatment with increasing concentrations of ethanolic myrrh extract (MY) significantly altered the Bay K8644 concentration-response relation in untreated preparations with regard to half maximal effective concentration (EC50) and maximum effect (Emax), whereby a right-ward shift of the concentration-response curve as well as a depression of maximum effect could be observed (Figure 22A; agonist only: EC50=6.0E-8, Emax=100.1 %; 0.15 mg/ml MY:

EC50=3.7E-7, Emax=96.14 %; 0.25 mg/ml MY: EC50=1.6E-7, Emax=57.13 %; 0.35 mg/ml MY: EC50=3.2E-7, Emax=15.86 %; Comparison of Fits [EC50; Emax] p=0.0008).

In inflamed preparations, the concentration-response curve of Bay K8644 after pre-treatment with increasing concentrations of ethanolic myrrh extract was likewise significantly altered (Figure 22B; agonist only: EC50=4.4E-8, Emax=100.3 %; 0.15 mg/ml MY: EC50=6.9E-7, Emax=127.1 %; 0.25 mg/ml MY: EC50=2.3E-7, Emax=98.70 %; 0.35 mg/ml MY: EC50=1.7E-6, Emax 25.78 %; Comparison of Fits [EC50; Emax] p=0.007).

Quantitative analysis of the shift of agonistic concentration-response curve representing competitive antagonistic effects was performed by calculating the pA_2 value using Schild plot. Equally, a quantification of non-competitive effects was done by calculation of the pD_2, based on the extent of Emax depression.

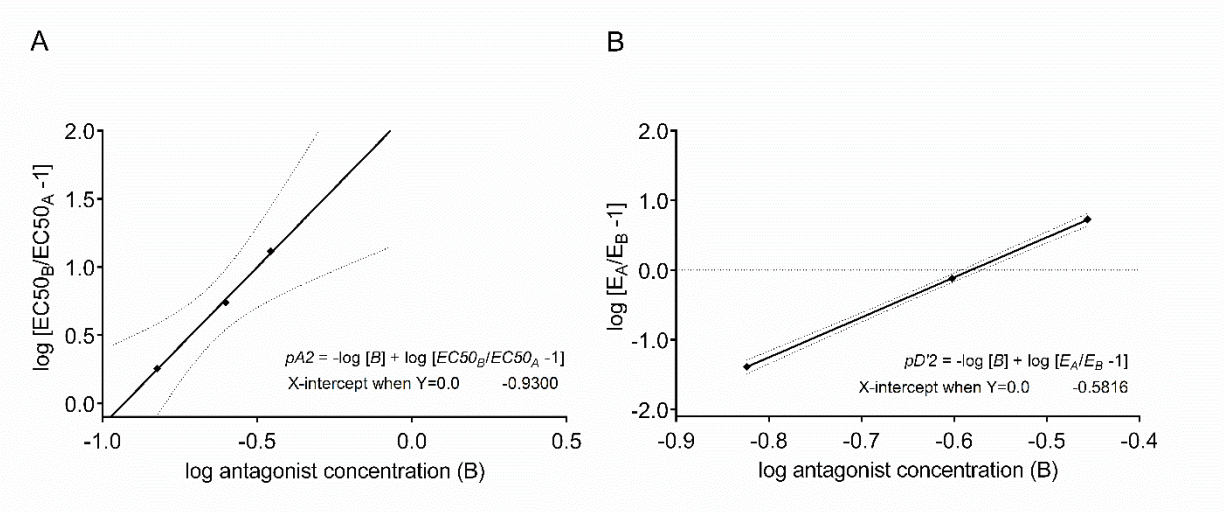

Figure 23: Schild analysis for different concentrations of ethanolic myrrh extract.
Analysis of EC50 shift (A) and Emax depression (B) by Schild analysis for increasing concentrations of ethanolic myrrh extract (0.15 - 0.35 mg/ml). Presented are linear regression and 95 % confidence interval (dotted line); pA2 = 0.93; pD2 = 0.56.

The graphic representation of these calculations is presented in Figure 23 for untreated preparations with a pA_2-value of 0.93. This corresponds to a concentration of 0.11 mg/ml ethanolic myrrh extract, which induced a 2-fold right-ward shift of the agonistic concentrations-response curve. The calculated pD_2-value of 0.58 corresponds to a concentration of 0.26 mg/ml ethanolic myrrh extract, which decreased the maximum effect induced by the agonist down to 50 %.

3.1.3 Spasmolytic activity of coffee charcoal extract

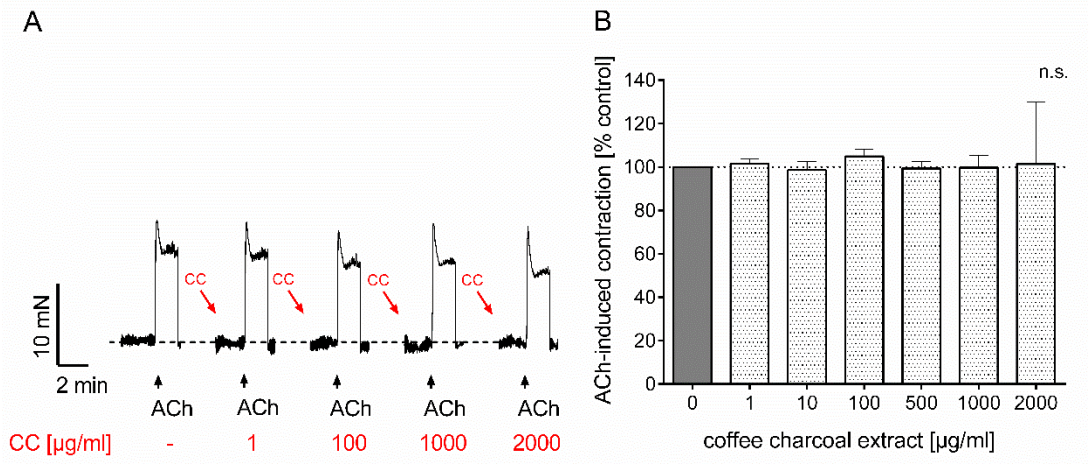

Figure 24: Influence of coffee charcoal extract (CC) on ACh-induced contractions in untreated ileum/jejunum preparations (A: representative original recording; B: statistical analysis). ACh (1 mM)-induced contractions were determined using isometric tension measurement. Coffee charcoal extract (0.35 – 700 µg/ml) was applied two minutes prior induction of contraction. Data are presented as mean ± SEM; the vertical line in (A) represents basal tone; n = 8.

No effect on ACh-induced contraction was observed, after coffee charcoal extract (0.35 – 700 µg/ml) was applied directly into the organ baths two minutes prior induction of contraction (Figure 24).

To investigate spasmolytic activity in inflamed tissue, inflammation was induced *in vitro* and ACh-induced contractions were assessed (Figure 25). Likewise, no effect on ACh-induced

contraction in inflamed tissue was observed, after coffee charcoal extract (0.35 – 700 µg/ml) application.

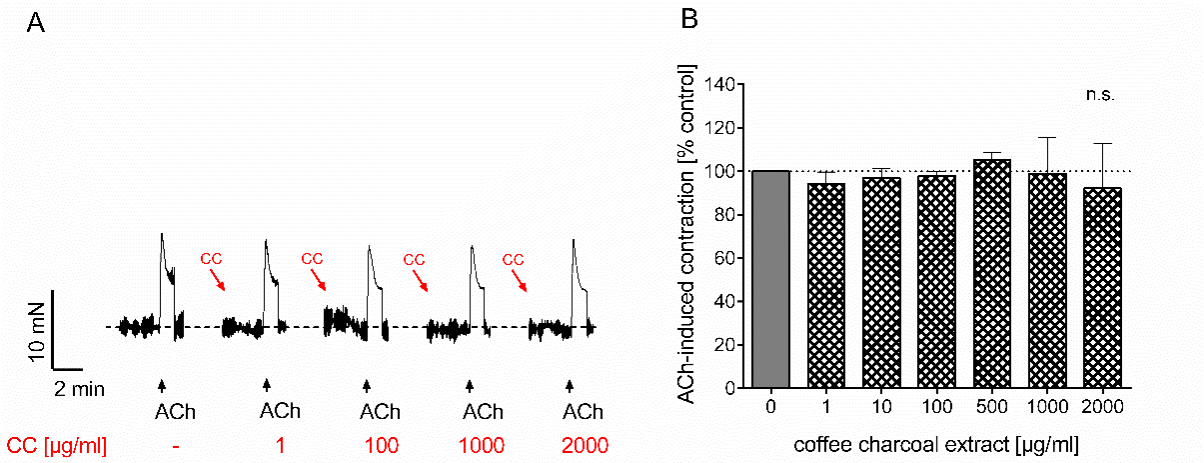

Figure 25: Influence of coffee charcoal extract (CC) on ACh-induced contractions in inflamed ileum/jejunum preparations (A: representative original recording; B: statistical analysis). Tonic contraction was induced in inflamed isolated rat ileum/jejunum preparations by application of ACh (1 mM). Coffee charcoal extract (0.35 – 700 µg/ml) was applied two minutes prior induction of contraction. Data are presented as mean ± SEM; the vertical line in (A) represents basal tone; n = 8.

3.2 Anti-inflammatory activity of Myrrhinil-Intest® components

3.2.1 Anti-inflammatory activity of chamomile flower extract

Effect of chamomile flower extract on TNBS-induced morphological damage

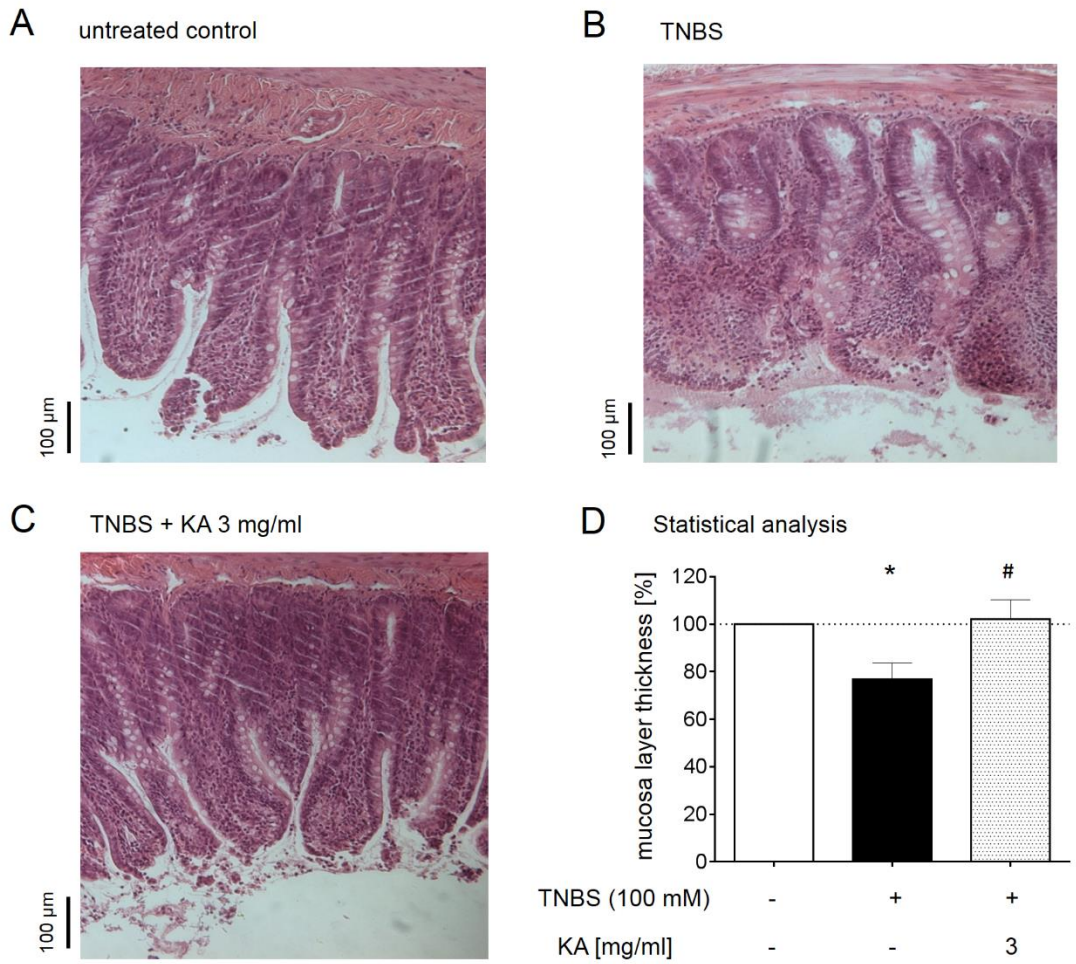

Figure 26: Effect of chamomile flower extract (3 mg/ml) on TNBS-induced morphological damage.
Rat ileum preparations were incubated with TNBS (100 mM) and chamomile flower extract (KA; 3 mg/ml) for 30 minutes and mucosa layer thickness was determined morphometrically after HE-staining. TNBS-incubation alone served as positive control. Presented are representative original images (A-C) and statistical analysis (D) with mean ± SEM; * $p<0.05$ vs. untreated control, # $p<0.05$ vs. TNBS; n = 4.

Incubation with TNBS (100 mM; 30 minutes) induced a morphological damage expressed by a decrease in mucosa layer thickness down to 76.86 % ± 6.8 compared to untreated control ($p<0.05$). Simultaneous incubation of TNBS and chamomile flower extract (KA, 3 mg/ml) inhibited the TNBS-induced decrease of mucosa layer thickness (Figure 26; 3 mg/ml KA: 102.1 % ± 8.1, $p<0.05$ vs. TNBS treatment).

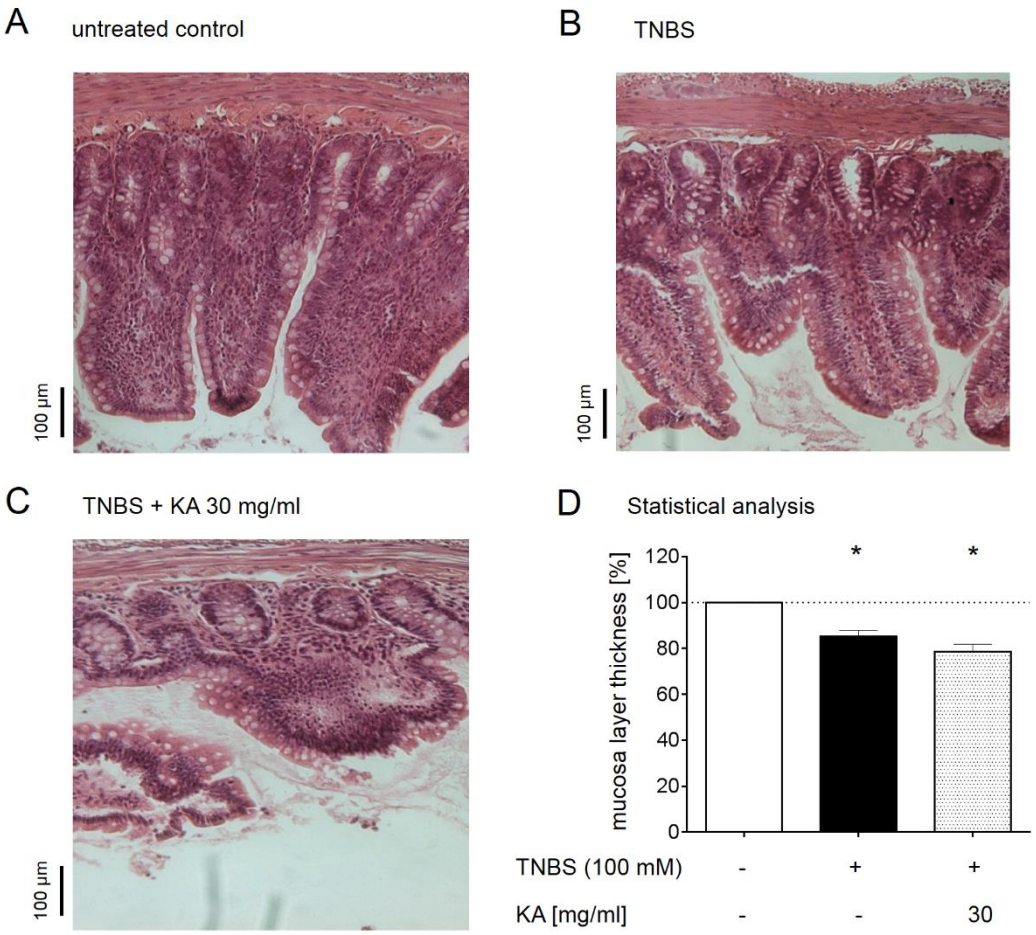

Figure 27: Effect of chamomile flower extract (30 mg/ml) on TNBS-induced morphological damage.
Rat ileum preparations were incubated with TNBS (10 mM) and chamomile flower extract (KA; 30 mg/ml) for 30 minutes and mucosa layer thickness was determined morphometrically after HE-staining. Single TNBS-incubation served as positive control. Presented are representative original images (A-C) and statistical analysis (D) with mean ± SEM; * $p<0.05$ vs. untreated control; n = 4.

In this series of experiments the incubation with TNBS (100 mM; 30 minutes) induced a morphological damage expressed by a decrease in mucosa layer thickness down to 85.26 % ± 2.6 compared to untreated control ($p<0.05$). Simultaneous incubation of TNBS and chamomile flower extract (KA, 30 mg/ml) led to a comparable decrease of mucosa layer thickness as observed with TNBS alone (Figure 27; 30 mg/ml KA: 78.66 % ± 3.2, $p<0.05$ vs. control).

Effect of chamomile flower extract on TNBS-induced contractility decrease

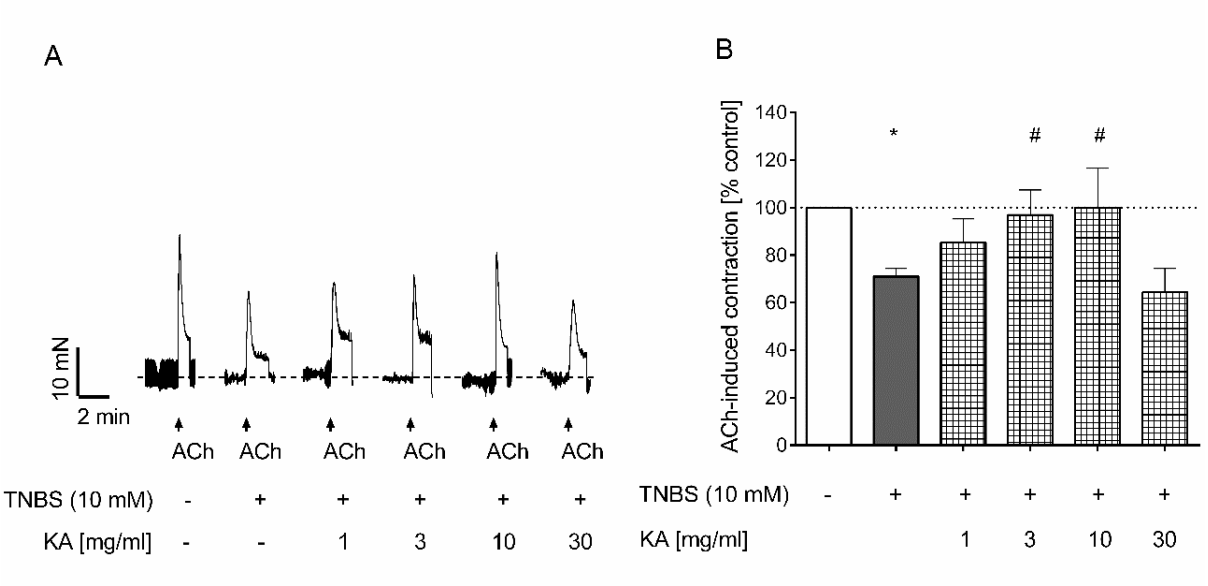

Figure 28: Effect of chamomile flower extract on TNBS-induced contractility decrease (A: representative original recording; B: statistical analysis).
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and chamomile flower extract (KA; 1 - 30 mg/ml) for 30 minutes and ACh-induced contractions were determined. TNBS-incubation alone served as positive control. Representative original recording (A) and statistical analysis (B) with mean ± SEM (n = 12); the vertical line in (A) represents basal tone; * $p<0.01$ vs. untreated control; # $p<0.05$ vs. TNBS.

Incubation with TNBS (10 mM; 30 minutes) induced a decrease in ACh-induced contraction down to 70.95 % ± 3.4 compared to untreated control ($p<0.05$). Simultaneous incubation of TNBS and chamomile flower extract (KA, 1 – 30 mg/ml) led to a significant inhibition of contractility decrease in concentrations of 3 and 10 mg/ml, resulting in an inverted U-shape concentration-effect relation (Figure 28; 1.0 mg/ml KA: 85.22±3.4; 3.0 mg/ml KA: 96.88±10.5, $p<0.05$ vs. TNBS; 10.0 mg/ml KA: 99.98±16.5, $p<0.05$ vs. TNBS; 30.0 mg/ml KA: 64.42±9.8).

Effect of chamomile flower extract on TNBS-induced TNFα gene expression

The influence of chamomile flower extract on TNBS-induced overexpression of TNFα-mRNA in rat small intestinal preparations is presented in Figure 29.

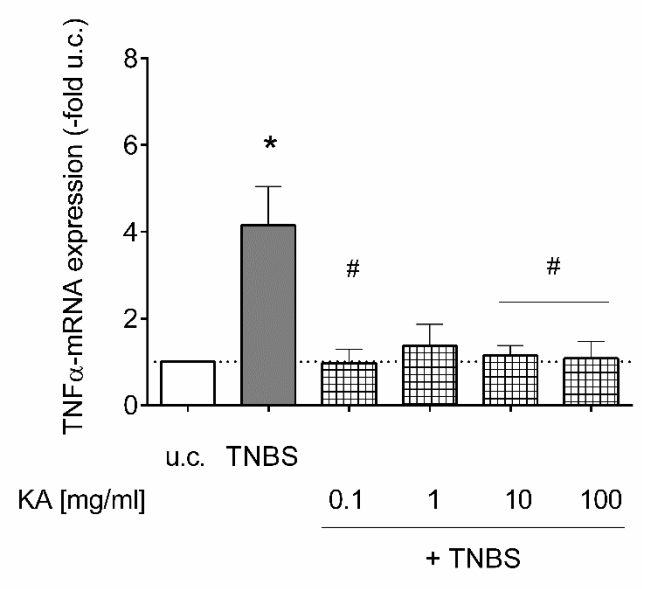

Figure 29: Effect of chamomile flower extract on TNBS-induced TNFα gene expression
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and chamomile flower extract (KA; 0.1 - 100 mg/ml) for 3 hours and the amount of TNFα-mRNA expression was analysed using real time PCR. Single TNBS-incubation served as positive control. Presented are mean ± SEM (n = 6) of TNFα-mRNA expression in relation to untreated control; * $p<0.01$ vs. untreated control; # $p<0.05$ vs. TNBS.

Incubation with TNBS (10 mM; 3 hours) induced a 4-fold increase in TNFα-mRNA expression compared to untreated control ($p<0.01$). Simultaneous incubation of TNBS and chamomile flower extract (KA, 0.1 – 100 mg/ml) led to a significant inhibition of the TNFα-mRNA expression (Figure 29; 0.1 mg/ml KA: 0.97±0.3, $p<0.05$; 1.0 mg/ml KA: 1.37±0.5; 10 mg/ml KA: 1.15±0.2, $p<0.05$; 100 mg/ml KA: 1.08±0.4, $p<0.05$; -fold compared to untreated control).

Effect of chamomile flower extract on LPS-induced TNFα release from THP-1 cells

To determine the influence of chamomile flower extract on LPS-induced TNFα release, LPS-stimulated (100 ng/ml), differentiated THP-1cells were incubated with chamomile flower extract (KA, 0.1 – 1000 µg/ml) for four hours. Subsequently, TNFα amount in cell supernatant was determined by ELISA. Differentiated THP-1 cells without LPS stimulation (u.c.), differentiated cells that were solely stimulated with LPS (LPS; 100 ng/ml) and differentiated cells that were treated with LPS and the anti-inflammatory glucocorticoid budesonide (Bud; 1 nM) were additionally tested as controls. TNFα release of LPS-stimulated THP-1 cells was set 100 %.

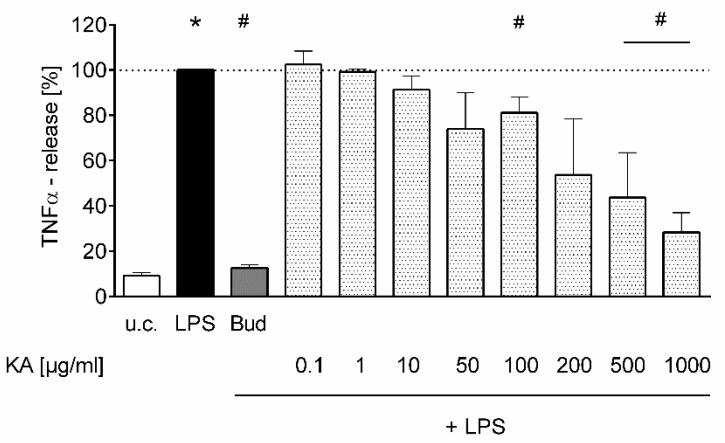

Figure 30: Effect of chamomile flower extract on LPS-induced TNFα release from THP-1 cells. Differentiated THP-1 cells were treated with LPS (100 ng/ml) and chamomile flower extract (KA; 0.1 – 1000 µg/ml) and incubated for four hours. TNFα release was determined by ELISA. Budesonide (Bud, 1 nM) served as positive control. Data are presented as mean ± SEM; * $p<0.05$ vs. untreated control (u.c.), # $p<0.01$ vs. LPS; n = 8-10.

LPS (100 ng/ml) significantly increased TNFα release in differentiated THP-1 cells (Figure 30; u.c.: 9.17 % ± 1.3; LPS: 100 %, $p<0.0001$). After simultaneous treatment with budesonide (1 nM), TNFα release was significantly decreased compared to single LPS stimulation (Figure 30; LPS: 100 %, Bud: 12.5 % ± 1.5; $p<0.0001$). DMSO in applied concentrations had no influence on TNFα release.

Chamomile flower extract led to a concentration-dependent decrease in TNFα release down to 28 % ± 8.7 (Figure 30; 0.1 µg/ml KA: 102.4±6.0; 1 µg/ml KA: 99.24±1.3; 10 µg/ml KA: 91.38±6.1; 50 µg/ml KA: 74.03±16.0; 100 µg/ml KA: 81.1±7.1, $p<0.01$; 200 µg/ml KA: 53.69±24.6; 500 µg/ml KA: 43.71±19.8, $p<0.01$; 1000 µg/ml KA: 28.28±8.7, $p<0.0001$; in % LPS-treatment).

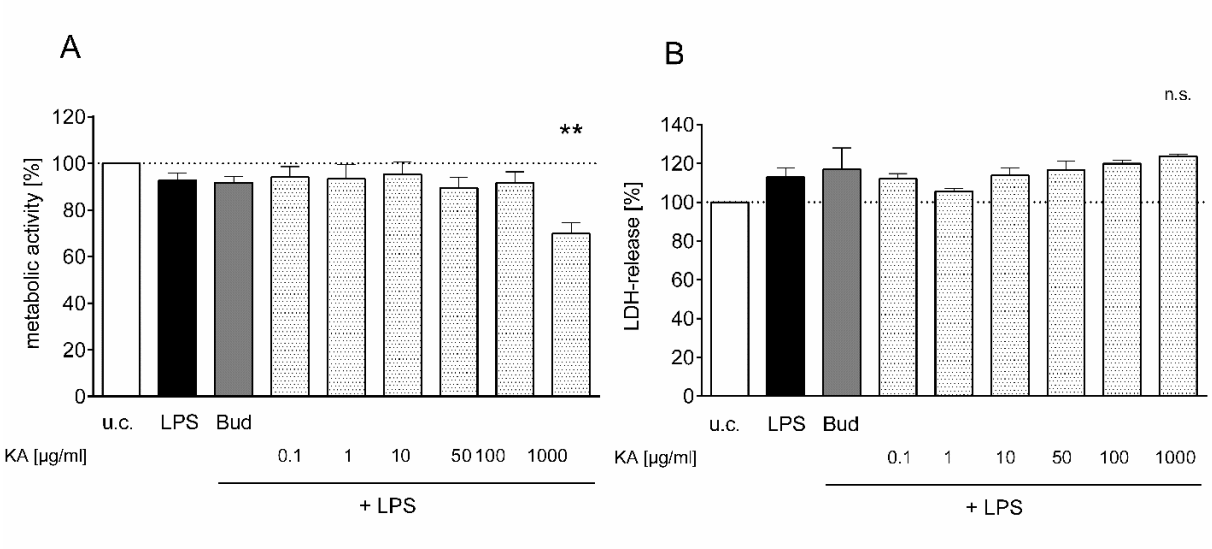

Figure 31: Effect of chamomile flower extract on metabolic activity (A) and LDH-release (B) of THP-1 cells.
Differentiated THP-1 cells were treated with LPS (100 ng/ml) and chamomile flower extract (KA; 0.1 – 1000 µg/ml) and incubated for four hours. Metabolic activity was determined by MTT-assay; LDH-assay was performed to determine LDH-release. Data are presented as mean ± SEM (n = 8-10); ** $p<0.01$ vs. untreated control (u.c.).

To evaluate the viability of the cells used for determination of TNFα release, MTT-assay and LDH-assay were performed after treatment with the respective substances (Figure 31).

Cell viability expressed as metabolic activity of differentiated THP-1 cells was not influenced after simultaneous incubation with LPS (100 ng/ml) and budesonide (1 nM). Increasing concentrations of chamomile flower extract did not alter cell viability in a concentration range of 0.1 – 100 µg/ml compared to untreated control. However, the highest concentration decreased metabolic activity to 69.84 % ± 4.8 ($p<0.0001$) compared to untreated control.

LDH-release was not influenced by treatment with LPS (100 ng/ml) or simultaneous treatment with budesonide (1 nM) or chamomile flower extract (0.1 – 1000 µg/ml).

To realise a distinction between the inhibition of TNFα release and influence of metabolic activity, a selectivity index was determined. Therefore, the concentration-response curves for TNFα release and metabolic activity were evaluated and half maximal inhibitory concentrations (IC50) were calculated (Figure 32).

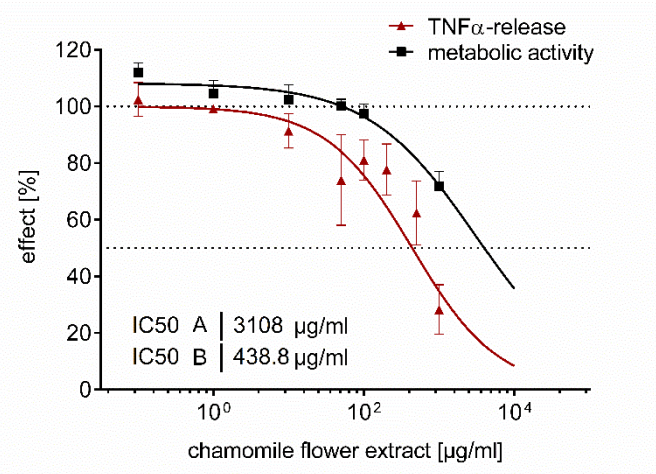

Figure 32: Concentration-response curves of chamomile flower extract effect on LPS-induced TNFα release and metabolic activity
To determine the selectivity index the concentration-response curves and IC50-values, for TNFα release (IC50 B) and metabolic activity (IC50 A) were ascertained by non-linear regression. Data are presented as mean ± SEM and non-linear regression curves for TNFα release (▲) and metabolic activity (■).

Chamomile flower extract reduced TNFα release with an IC50-value of 438.8 µg/ml and metabolic activity with an IC50-value of 3106 µg/ml. The resulting selectivity index (SI = 7.08) indicates that reduction of metabolic activity occurs in 7-fold higher concentrations compared to reduction of TNFα release.

3.2.2 Anti-inflammatory activity of myrrh

Effect of aqueous and ethanolic myrrh extract on TNBS-induced morphological damage

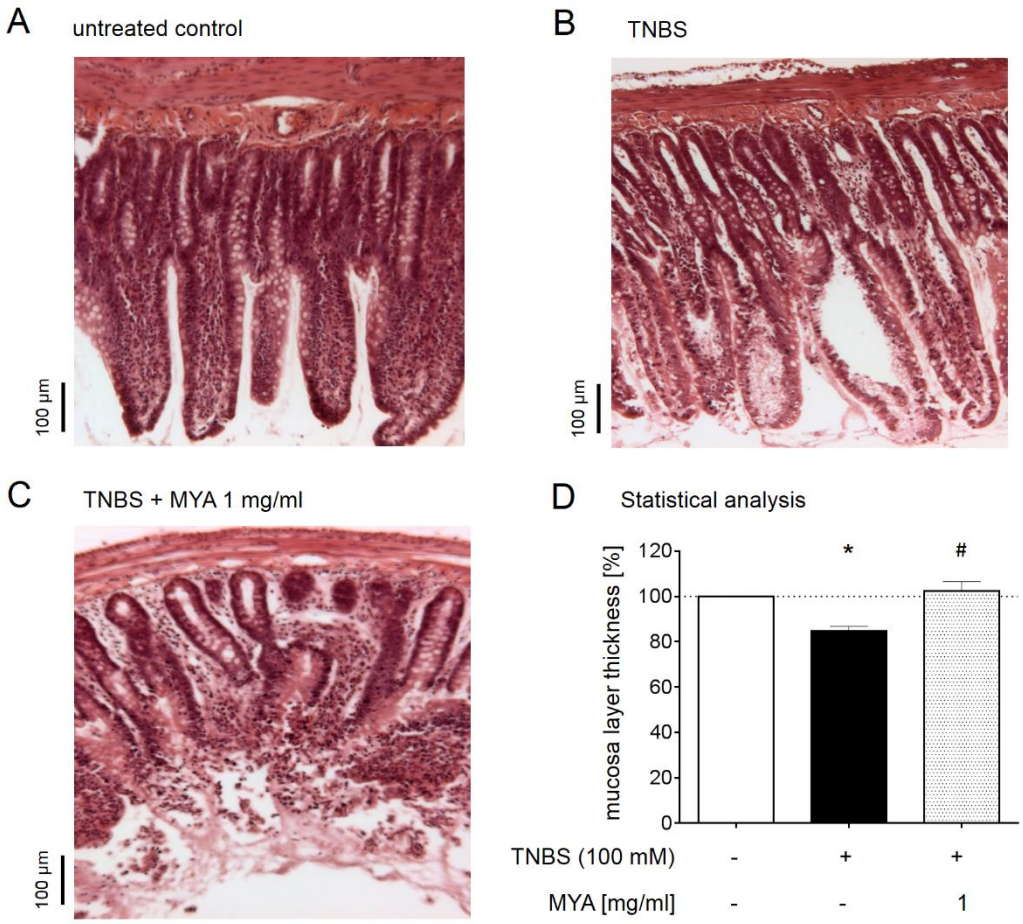

Figure 33: Effect of aqueous myrrh extract on TNBS-induced morphological damage.
Rat ileum preparations were incubated with TNBS (100 mM) and aqueous myrrh extract (MYA; 1000 µg/ml) for 30 minutes and mucosa layer thickness was determined morphometrically after HE-staining. TNBS-incubation alone served as positive control. Presented are representative original images (A-C) and statistical analysis (D) with mean ± SEM; * $p<0.0001$ vs. untreated control, # $p<0.01$ vs. TNBS; n = 4.

Incubation with TNBS (100 mM; 30 minutes) induced a morphological damage expressed by a decrease in mucosa layer thickness down to 84.79 % ± 2.0 compared to untreated control ($p<0.0001$). Simultaneous incubation of aqueous myrrh extract (MYA, 1 mg/ml) inhibited the TNBS-induced decrease of mucosa layer thickness (Figure 33; 1 mg/ml MYA: 102.4 % ± 4.1, $p<0.01$ vs. TNBS treatment).

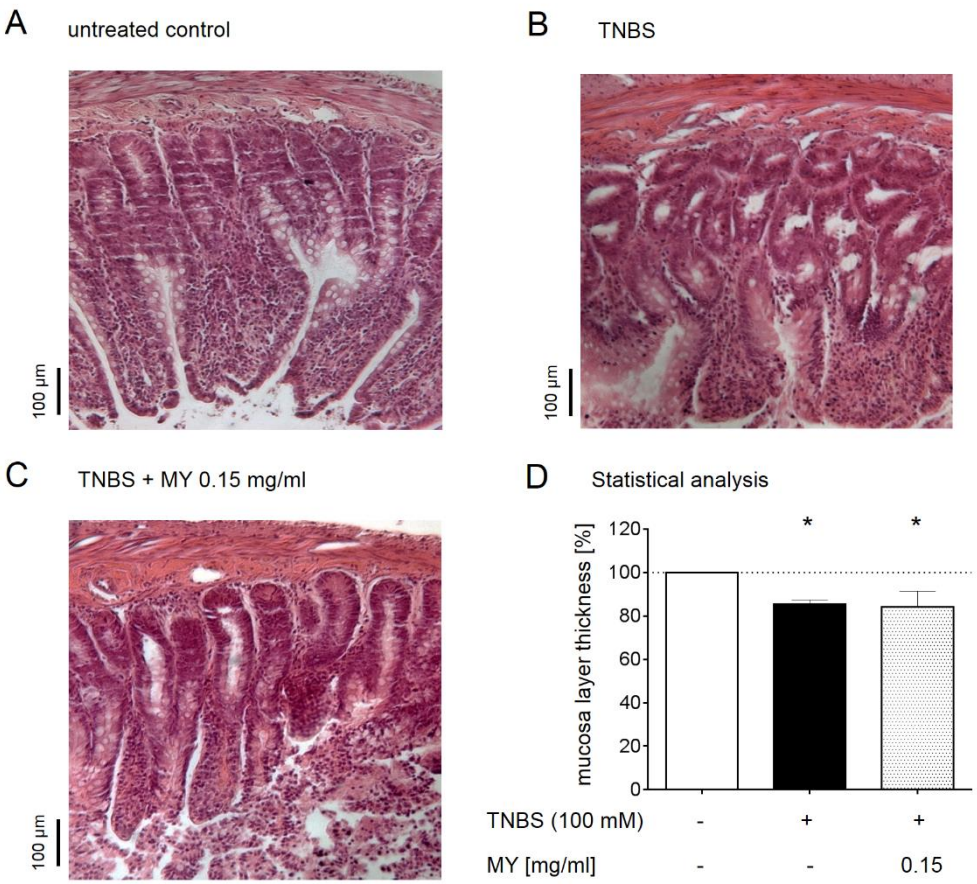

Figure 34: Effect of ethanolic myrrh extract on TNBS-induced morphological damage.
Rat ileum preparations were incubated with TNBS (100 mM) and ethanolic myrrh extract (MY; 150 µg/ml) for 30 minutes and mucosa layer thickness was determined morphometrically after HE-staining. TNBS-incubation alone served as positive control. Presented are representative original images (A-C) and statistical analysis (D) with mean ± SEM; * $p<0.01$; n = 4.

The influence of ethanolic myrrh extract (0.15 mg/ml) on TNBS-induced morphological damage in rat ileum preparations is presented in Figure 34. Incubation with TNBS (100 mM; 30 minutes) induced a morphological damage expressed by a decrease in mucosa layer thickness down to 85.57 % ± 1.9 compared to untreated control ($p<0.0001$). Simultaneous incubation of ethanolic myrrh extract (MY, 0.15 mg/ml) led to a comparable decrease of mucosa layer thickness (Figure 34; 0.15 mg/ml MY: 84.16 % ± 7.3, $p<0.001$).

Effect of ethanolic myrrh extract on TNBS-induced contractility decrease

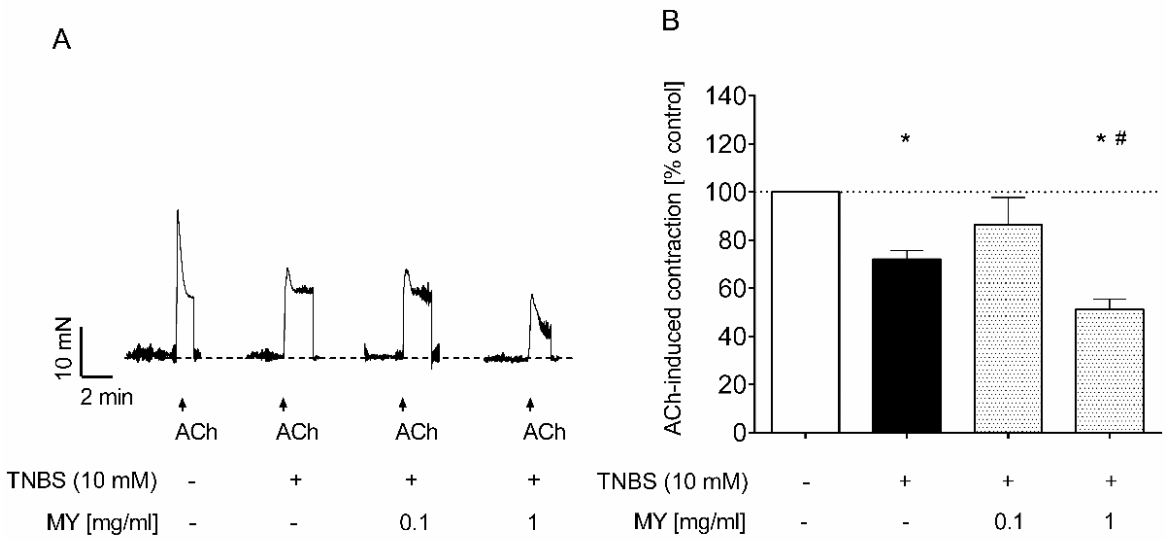

Figure 35: Effect of ethanolic myrrh extract on TNBS-induced contractility decrease (A: representative original recording; B: statistical analysis).
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and ethanolic myrrh extract (MY; 0.1 – 1 mg/ml) for 30 minutes and ACh-induced contractions were determined. TNBS-incubation alone served as positive control. Presented is a representative original recording (A) and statistical analysis (B) with mean ± SEM (n = 12); the vertical line in (A) represents basal tone; * $p<0.01$ vs. untreated control, # $p<0.05$ vs. TNBS.

In this series of experiments incubation with TNBS (10 mM; 30 minutes) induced a decrease in ACh-induced contraction down to 71.99 % ± 3.6 compared to untreated control ($p<0.01$). Simultaneous incubation of ethanolic myrrh extract in a concentration of 0.1 mg/ml did not significantly alter ACh-induced contractions compared to control (Figure 35; 0.1 µg/ml MY: 86.38 % ± 11.35). However, 1.0 mg/ml ethanolic myrrh extract led to a further decrease in ACh-induced contraction compared to single TNBS-treatment (Figure 35; 1.0 µg/ml MY: 53.48 % ± 4.6, $p<0.05$ vs. TNBS).

Effect of aqueous myrrh extract on TNBS-induced contractility decrease

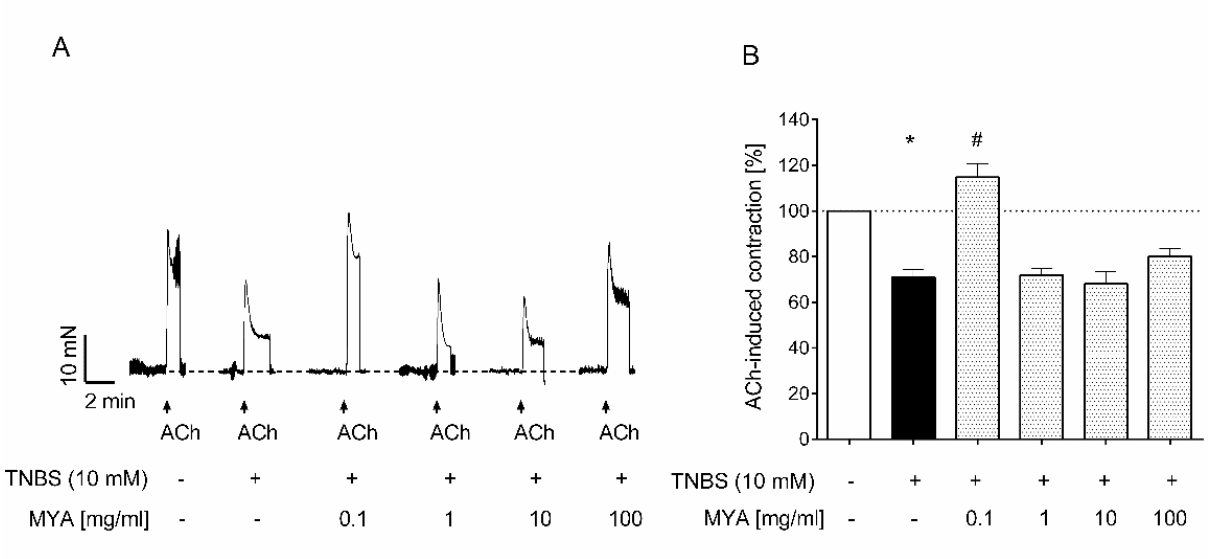

Figure 36: Effect of aqueous myrrh extract on TNBS-induced contractility decrease (A: representative original recording; B: statistical analysis).
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and aqueous myrrh extract (MYA; 0.1 – 1 mg/ml) for 30 minutes and ACh-induced contractions were determined. TNBS-incubation alone served as positive control. Presented is a representative original recording (A) and statistical analysis (B) with mean ± SEM; the vertical line in (A) represents basal tone; n = 12.

The influence of aqueous myrrh extract on TNBS-induced contractility decrease is presented in Figure 36. Incubation with TNBS (10 mM; 30 minutes) induced a decrease in ACh-induced contraction down to 70.95 % ± 3.6 compared to untreated control ($p<0.001$). Simultaneous incubation of aqueous myrrh extract in a concentration of 1 – 100 mg/ml did not significantly alter ACh-induced contractility compared to control (Figure 36; 1 mg/ml MYA: 71.84±2.9; 10 mg/ml MYA: 68.06±5.4; 100 mg/ml MYA: 80.03±3.6, in % control). However, the lowest concentration of aqueous myrrh extract tested, inhibited the TNBS-induced reduction of ACh-induced contraction (Figure 36; 0.1 mg/ml MYA: 114.8±5.7, $p<0.001$ vs. TNBS).

Effect of ethanolic myrrh extract on TNBS-induced TNFα gene expression

The influence of ethanolic myrrh extract on TNBS-induced overexpression of TNFα-mRNA in rat small intestinal preparations is presented in Figure 37.

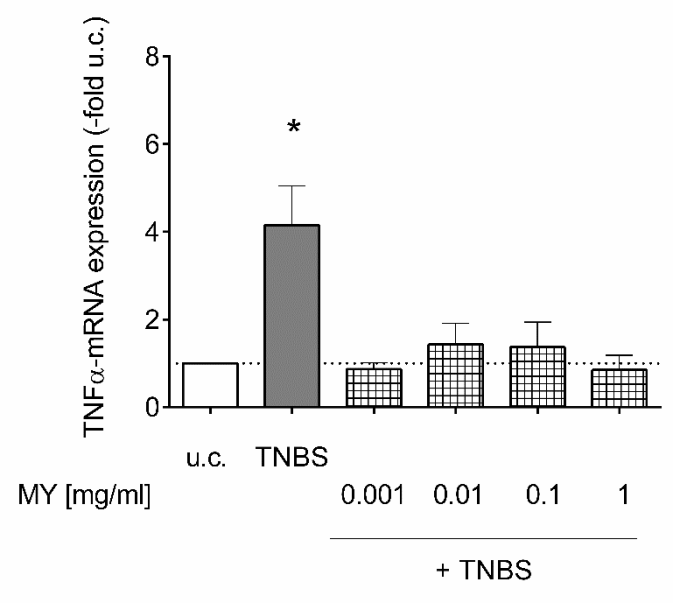

Figure 37: Effect of ethanolic myrrh extract on TNBS-induced TNFα gene expression
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and ethanolic myrrh extract (MY; 0.1 - 100 mg/ml) for 3 hours and the amount of TNFα-mRNA expression was analysed using real time PCR. TNBS-incubation alone served as positive control. Presented are mean ± SEM (n = 4) of TNFα-mRNA expression in relation to untreated control; * $p<0.01$ vs. untreated control.

Incubation with TNBS (10 mM; 3 hours) induced a 4-fold increase in TNFα-mRNA expression compared to untreated control ($p<0.01$). Simultaneous incubation of TNBS and ethanolic myrrh extract (0.001 – 1 mg/ml) resulted in mRNA-expression levels below that induced by TNBS, however no statistically significant reduction in TNFα-mRNA expression compared to TNBS effect could be observed. (Figure 37; 0.001 mg/ml MY: 0.87±0.1; 0.01 mg/ml MY: 1.43±0.5; 0.1 mg/ml MY: 1.36±0.3; 1 mg/ml MY: 0.85±0.3; -fold compared to untreated control).

Effect of aqueous myrrh extract on TNBS-induced TNFα gene expression

The influence of aqueous myrrh extract on TNBS-induced overexpression of TNFα-mRNA in rat small intestinal preparations is presented in Figure 38.

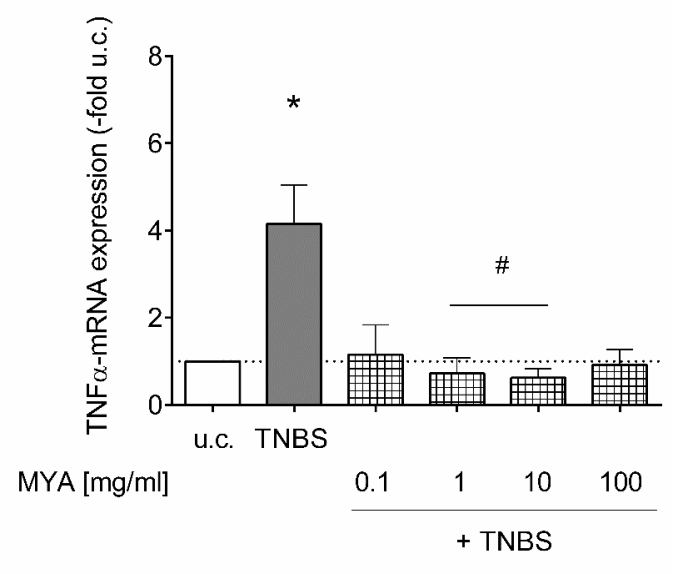

Figure 38: Effect of aqueous myrrh extract on TNBS-induced TNFα gene expression
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and aqueous myrrh extract (MY; 0.1 - 100 mg/ml) for 3 hours and the amount of TNFα-mRNA expression was analysed using real time PCR. TNBS-incubation alone served as positive control. Presented are mean ± SEM (n = 4) of TNFα-mRNA expression in relation to untreated control; * $p<0.01$ vs. untreated control, # $p<0.05$ vs. TNBS.

Incubation with TNBS (10 mM; 3 hours) induced a 4-fold increase in TNFα-mRNA expression compared to untreated control ($p<0.01$). Simultaneous incubation of TNBS and aqueous myrrh extract (MYA, 0.1 – 100 mg/ml) led to a significant inhibition of the TNFα-mRNA expression (Figure 38; 0.1 mg/ml MYA: 1.16±0.7; 1.0 mg/ml MYA: 0.72±0.4, $p<0.05$; 10 mg/ml MYA: 0.62±0.2, $p<0.05$; 100 mg/ml MYA: 0.92±0.4; -fold compared to untreated control).

Effect of ethanolic myrrh extract on LPS-induced TNFα release

To determine the influence of ethanolic myrrh extract on LPS-induced TNFα release, LPS-stimulated (100 ng/ml), differentiated THP-1cells were incubated with ethanolic myrrh extract (MY, 0.1 – 200 µg/ml) for four hours. Subsequently, TNFα amount in cell supernatant was determined by ELISA. Differentiated THP-1 cells without LPS stimulation (u.c.), differentiated cells that were solely stimulated with LPS (LPS; 100 ng/ml) and differentiated cells that were treated with LPS and the anti-inflammatory glucocorticoid budesonide (Bud; 1 nM) were additionally tested as controls. TNFα release of LPS-stimulated THP-1 cells was set 100 %.

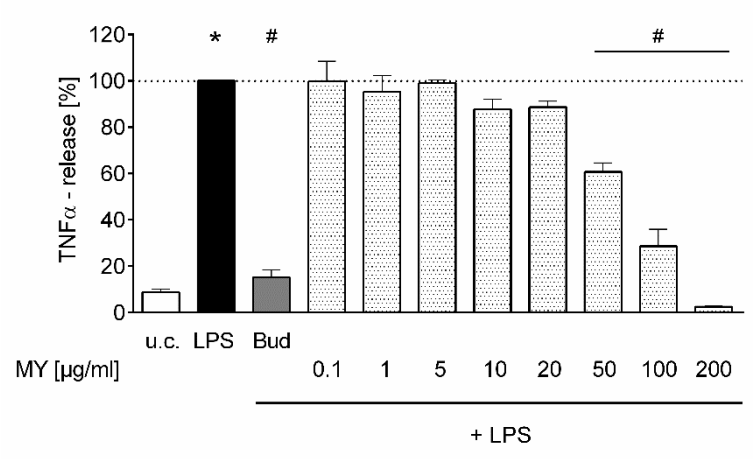

Figure 39: Effect of ethanolic myrrh extract on LPS-induced TNFα release from THP-1 cells. Differentiated THP-1 cells were treated with LPS (100 ng/ml) and ethanolic myrrh extract (MY; 0.1 – 200 µg/ml) and incubated for four hours. TNFα release was determined by ELISA. Budesonide (Bud, 1 nM) served as positive control. Data are presented as mean ± SEM; * $p<0.05$ vs. untreated control (u.c.), # $p<0.001$ vs. LPS, n = 8-10.

The influence of ethanolic myrrh extract (MY, 0.1 – 200 µg/ml) on LPS-induced TNFα release from THP-1 cells is presented in Figure 39. LPS (100 ng/ml) significantly increased TNFα release in differentiated THP-1 cells (Figure 39; u.c.: 8.73 % ± 1.4; LPS: 100 %, $p<0.0001$). After simultaneous treatment with budesonide (1 nM), TNFα release was significantly decreased compared to single LPS stimulation (Figure 39; LPS: 100 %, Bud: 15.15 % ± 3.2; $p<0.0001$). DMSO in applied concentrations had no influence on TNFα release.

Ethanolic myrrh extract led to a concentration-dependent decrease in TNFα release down to 2 % ± 0.3 (Figure 39; 0.1 µg/ml MY: 99.76±8.8; 1.0 µg/ml MY: 95.3±7.0; 5.0 µg/ml MY: 99.03±1.5; 10.0 µg/ml MY: 87.79±4.4; 20 µg/ml MY: 88.69±2.6; 50 µg/ml MY: 60.69±3.9, $p<0.0001$; 100 µg/ml MY: 28.57±7.4, $p<0.0001$; 200 µg/ml MY: 2.45±0.3, $p<0.0001$; in % LPS-treatment).

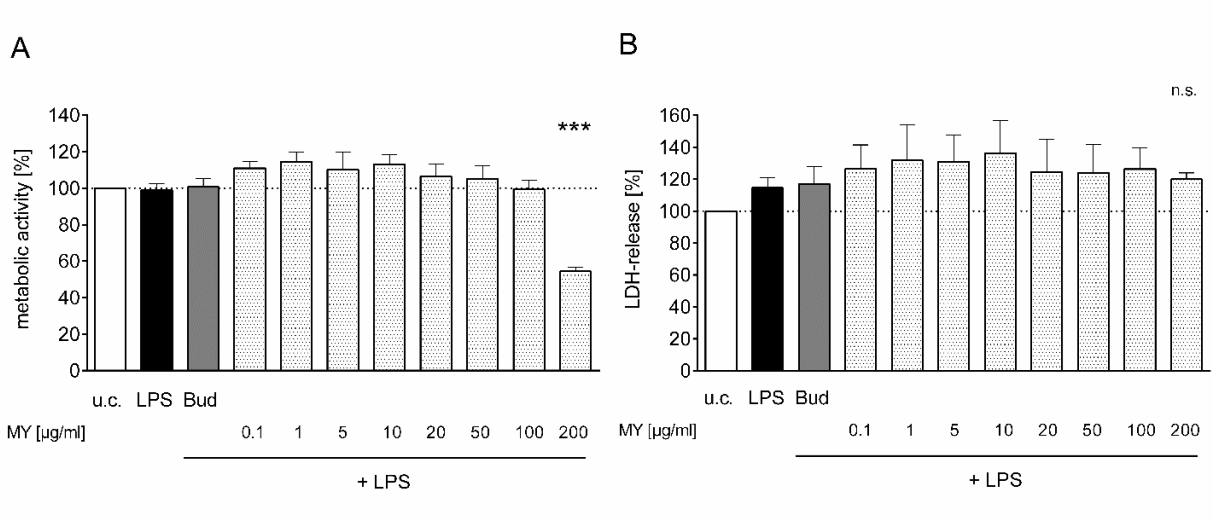

Figure 40: Effect of ethanolic myrrh extract on metabolic activity (A) and LDH-release (B) of THP-1 cells.
Differentiated THP-1 cells were treated with LPS (100 ng/ml) and ethanolic myrrh extract (MY; 0.1 – 200 µg/ml) and incubated for four hours. Metabolic activity was determined by MTT-assay; LDH-assay was performed to determine LDH-release. Data are presented as mean ± SEM (n = 8-10); *** $p<0.001$ vs. untreated control (u.c.).

To evaluate the viability of the cells used for determination of TNFα release, MTT-assay and LDH-assay were performed after treatment with the respective substances (Figure 40).

Cell viability expressed as metabolic activity of differentiated THP-1 cells was not influenced after simultaneous incubation with LPS (100 ng/ml) and budesonide (1 nM). Increasing concentrations of ethanolic myrrh extract did not alter cell viability in a concentration range of 0.1 – 100 µg/ml compared to untreated control. However, the highest concentration decreased metabolic activity to 48.25 % ± 2.2 ($p<0.001$) compared to untreated control.

LDH-release was not influenced by treatment with LPS (100 ng/ml) or simultaneous treatment with budesonide (1 nM) or ethanolic myrrh extract (0.1 – 1000 µg/ml).

To realise a distinction between the inhibition of TNFα release and influence of metabolic activity, a selectivity index was determined. Therefore, the concentration-response curves for TNFα release and metabolic activity were evaluated and half maximal inhibitory concentrations (IC50) were calculated (Figure 41).

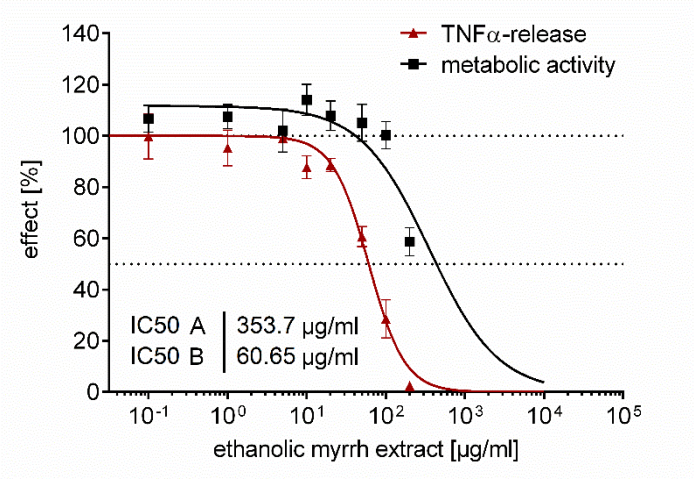

Figure 41: Concentration-response curves of ethanolic myrrh extract on LPS-induced TNFα release and metabolic activity
To determine the selectivity index the concentration-response curves and IC50-values, for TNFα release (IC50 B) and metabolic activity (IC50 A) were ascertained by non-linear regression. Data are presented as mean ± SEM (n = 8-10) and non-linear regression curves for TNFα release (▲) and metabolic activity (■).

Ethanolic myrrh extract reduced the TNFα release with an IC50-value of 60.65 µg/ml and the metabolic activity with an IC50-value of 353.7 µg/ml. The resulting selectivity index (SI = 5.8) indicates that reduction of metabolic activity occurs in 6-fold higher concentrations compared to reduction of TNFα release.

Effect of aqueous myrrh extract on LPS-induced TNFα release

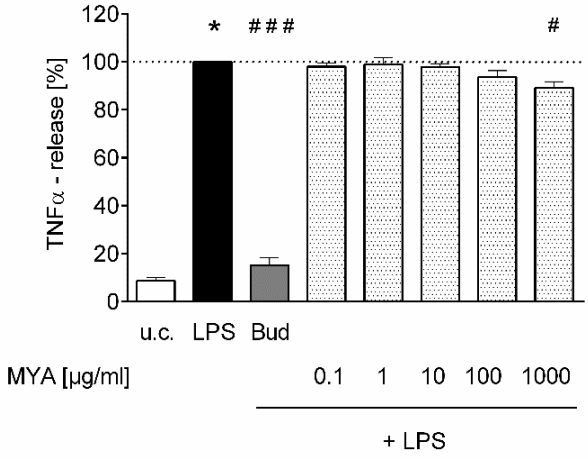

Figure 42: Effect of aqueous myrrh extract on LPS-induced TNFα release from THP-1 cells.
Differentiated THP-1 cells were treated with LPS (100 ng/ml) and aqueous myrrh extract (MYA; 0.1 – 1000 µg/ml) and incubated for four hours. TNFα release was determined by ELISA. Budesonide (Bud, 1 nM) served as positive control. Data are presented as mean ± SEM; * $p<0.05$ vs. untreated control (u.c.), # # # $p<0.001$ vs. LPS, # $p<0.05$ vs. LPS; n = 8-10.

LPS (100 ng/ml) significantly increased TNFα release in differentiated THP-1 cells (Figure 42; u.c.: 8.73 % ± 1.4; LPS: 100 %, p<0.0001). After simultaneous treatment with budesonide (1 nM) TNFα release was significantly decreased compared to single LPS stimulation (Figure 42; LPS: 100 %, Bud: 15.15 % ± 3.2; p<0.0001).

Aqueous myrrh extract in concentrations up to 100 µg/ml did not influence TNFα release compared to sole LPS stimulation. However, in the highest concentration tested it significantly decreased LPS-induced TNFα release to a comparably small extent (Figure 42; 0.1 mg/ml MYA: 97.98±1.5; 1 µg/ml MYA: 98.92±2.7; 10 µg/ml MYA: 97.85±1.4; 100 µg/ml MYA: 93.68±2.6; 1000 µg/ml MYA: 89.24±2.5, p<0.05; in % LPS-treatment).

To evaluate the viability of the cells used for determination of TNFα release, MTT-assay and LDH-assay were performed after treatment with the respective substances (Figure 43).

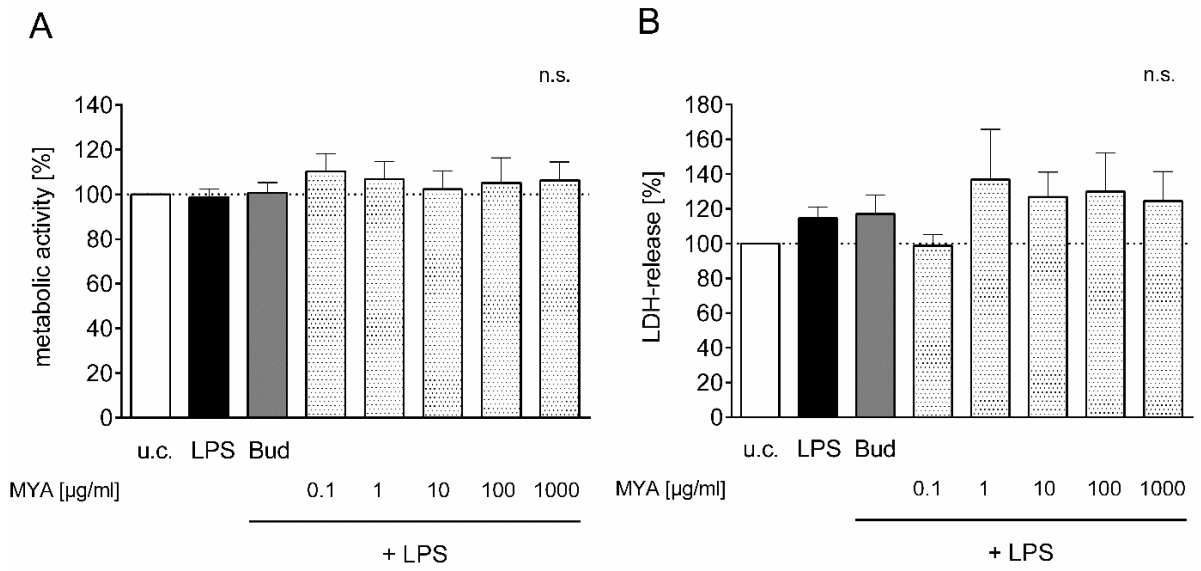

Figure 43: Effect of aqueous myrrh extract on metabolic activity (A) and LDH-release (B) of THP-1 cells.
Differentiated THP-1 cells were treated with LPS (100 ng/ml) and aqueous myrrh extract (MYA; 0.1 – 1000 µg/ml) and incubated for four hours. Metabolic activity was determined by MTT-assay; LDH-assay was performed to determine LDH-release. Data are presented as mean ± SEM; n = 8-10.

Cell viability expressed as metabolic activity of differentiated THP-1 cells was not influenced after simultaneous incubation with LPS (100 ng/ml) and budesonide (1 nM).

Aqueous myrrh extract in the concentrations tested did not influence metabolic activity of the cells.

Treatment with LPS (100 ng/ml) or simultaneous treatment with budesonide (1 nM) or aqueous myrrh extract (0.1 – 1000 µg/ml) did not alter LDH-release compared to untreated control.

Effect of aqueous myrrh extract on basal TNFα release

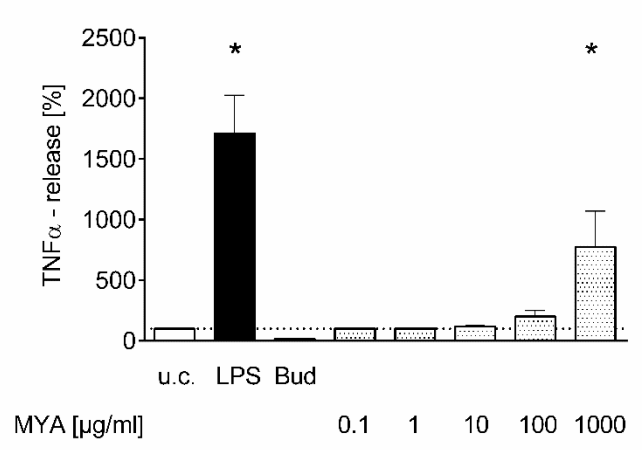

Figure 44: Effect of aqueous myrrh extract on basal TNFα release from THP-1 cells.
Differentiated THP-1 cells were treated with aqueous myrrh extract (MYA; 0.1 – 1000 µg/ml) and incubated for four hours. Budesonide (Bud, 1 nM) served as positive control. TNFα release was determined by ELISA. Data are presented as mean ± SEM; * $p<0.0001$ vs. untreated control (u.c.); n = 8.

To determine the effect of aqueous myrrh extract on basal TNFα release, differentiated THP-1 cells were incubated with aqueous myrrh extract (MYA, 0.1 – 1000 µg/ml) for four hours and TNFα in the cell supernatant was quantified. LPS as positive control induced a TNFα release of 1712 % ± 315 compared to untreated control. Aqueous myrrh extract in concentrations up to 100 µg/ml did not influence basal TNFα release compared to untreated control. However, the highest concentration led to an increase in TNFα release (Figure 44; 0.1 µg/ml MYA: 101.1±5.1; 1.0 µg/ml MYA: 100.9±4.0; 10 µg/ml MYA: 117.4±7.9; 100 µg/ml MYA: 201.2±47.35; 1000 µg/ml MYA: 774.8±294.7, $p<0.0001$; in % untreated control).

Effect of ethanolic myrrh extract on LPS-induced NO release

The following experiments were kindly allowed to be performed at the Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, in the laboratories of Prof. A. Hensel.

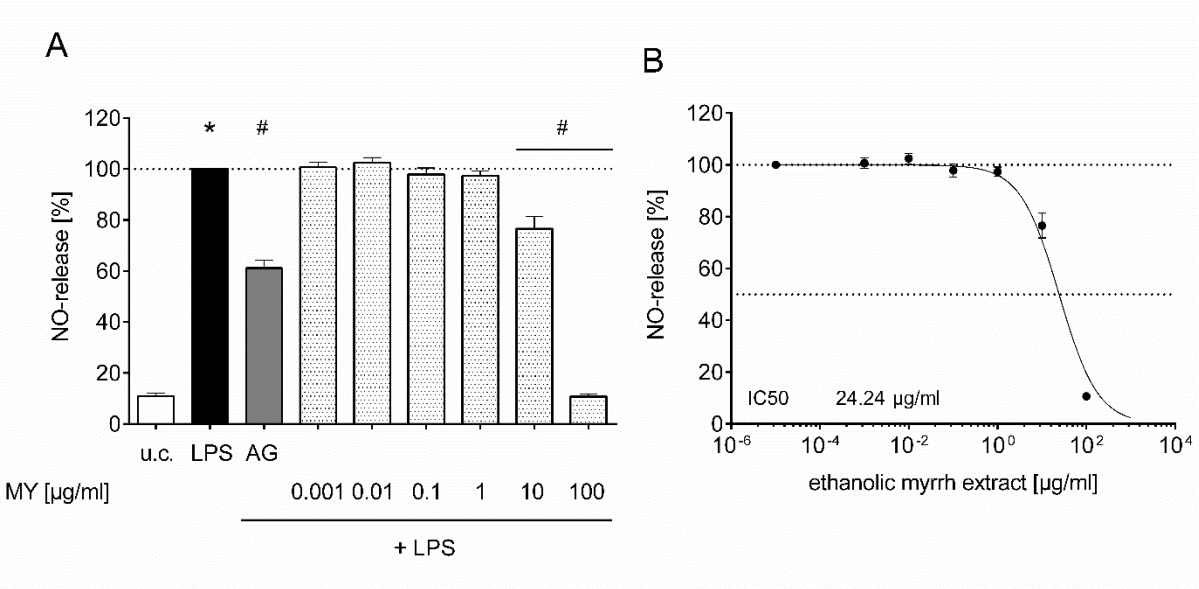

Figure 45: Effect of ethanolic myrrh extract on LPS-induced nitric oxide (NO) release from RAW 264.7 cells
RAW 264.7 macrophages were treated with LPS (100 ng/ml) and ethanolic myrrh extract (MY; 0.1 – 1000 µg/ml) and incubated for 24 hours. NO release was determined colorimetrically by Griess reaction. Aminoguanidine (AG, 1 nM) served as positive control. Presented are statistical analysis [A] and concentration-response curve [B]. Data are presented as mean ± SEM and non-linear regression; * $p<0.05$ vs. untreated control (u.c.); # $p<0.001$ vs. LPS; n = 6.

LPS (100 ng/ml) significantly increased NO release from RAW 264.7 macrophages (Figure 45; u.c.: 10.93 % ± 1.3; LPS: 100 %, $p<0.001$). After simultaneous treatment with the inducible nitric oxide synthase inhibitor aminoguanidine (1 nM), NO release was significantly decreased compared to single LPS stimulation (Figure 45; LPS: 100 %, aminoguanidine: 61.15 % ± 3.2; $p<0.001$). DMSO in applied concentrations had no influence on NO release.

Ethanolic myrrh extract led to a concentration-dependent decrease in NO release down to 11 % ± 1.0 with a half maximal inhibitory concentration (IC50) of 24.24 µg/ml (Figure 45; 0.001 µg/ml MY: 100.7±2.1; 0.01 µg/ml MY: 102.5±2.0; 0.1 µg/ml MY: 97.87±2.6; 1 µg/ml MY: 97.42±1.8; 10 µg/ml MY: 76.58±4.8, $p<0.001$; 100 µg/ml MY: 10.69±1.0, $p<0.001$; in % LPS-treatment).

Effect of aqueous myrrh extract on LPS-induced NO release

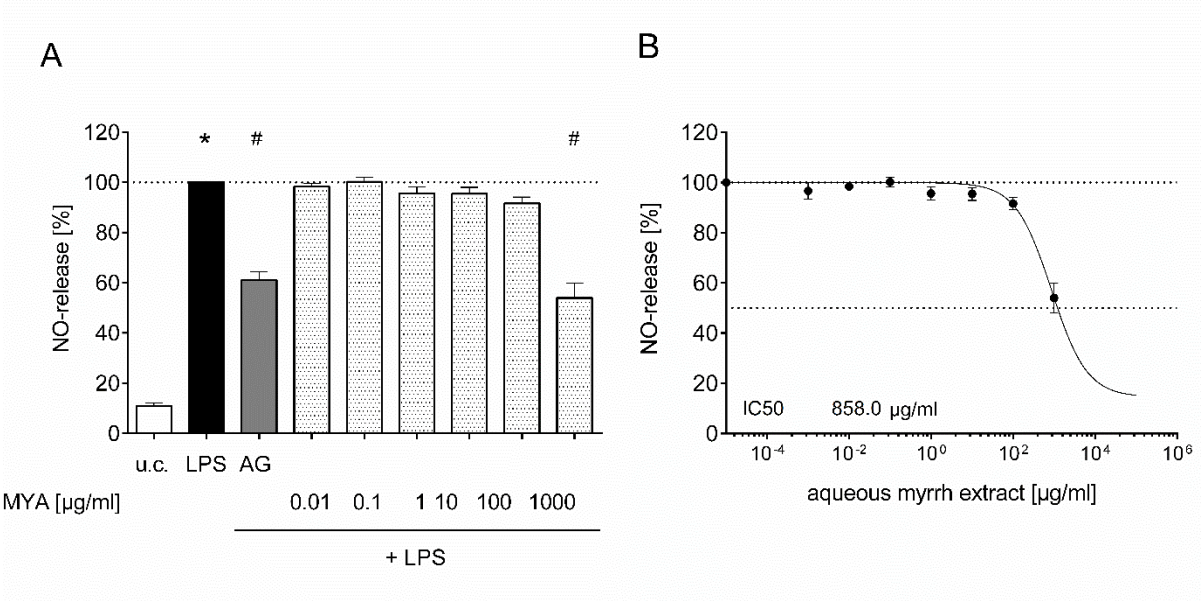

Figure 46: Effect of aqueous myrrh extract on LPS-induced nitric oxide (NO) release from RAW 264.7 cells
RAW 264.7 macrophages were treated with LPS (100 ng/ml) and aqueous myrrh extract (MYA; 0.1 – 1000 µg/ml) and incubated for 24 hours. NO release was determined colorimetrically by Griess reaction. Aminoguanidine (AG, 1 nM) served as positive control. Presented are statistical analysis [A] and concentration-response curve [B]. Data are presented as mean ± SEM and non-linear regression; * $p<0.05$ vs. untreated control (u.c.); # $p<0.001$ vs. LPS; n = 6.

LPS (100 ng/ml) significantly increased NO release from RAW 264.7 macrophages (Figure 46; u.c.: 10.93 % ± 1.3; LPS: 100 %, $p<0.001$). After simultaneous treatment with the inducible nitric oxide synthase inhibitor aminoguanidine (1 nM), NO release was significantly decreased compared to single LPS stimulation (Figure 46; LPS: 100 %, aminoguanidine: 61.15 % ± 3.2; $p<0.001$).

Aqueous myrrh extract in concentrations up to 100 µg/ml did not influence LPS-stimulated NO release. However, the highest concentration induced a decrease of released NO (Figure 46; 0.01 µg/ml MYA: 96.65±3.3; 0.1 µg/ml MYA: 98.46±1.2; 1 µg/ml MYA: 100.2±2.0; 10 µg/ml MYA: 95.68±2.6; 10 µg/ml: 95.48±2.6; 100 µg/ml: 91.61±2.6; 1000 µg/ml MYA: 53.98±6.0, $p<0.001$ in % untreated control; IC50: 858.0 µg/ml).

Effect of aqueous myrrh extract on basal NO release

To determine the effect of aqueous myrrh extract on basal NO release, RAW 264.7 cells were incubated with aqueous myrrh extract (MYA; 0.01 – 1000 µg/ml) for 48 hours and NO release was quantified colorimetrically by Griess reaction.

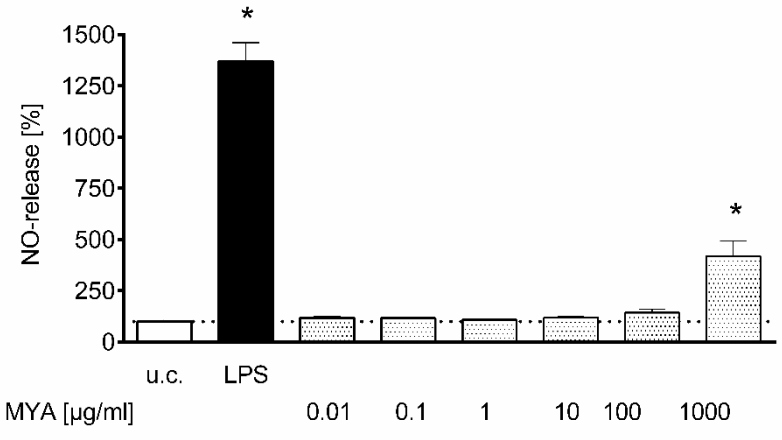

Figure 47: Effect of aqueous myrrh extract on basal NO release from RAW 264.7 cells.
RAW 264.7 cells were treated with aqueous myrrh extract (MYA; 0.01 – 1000 µg/ml) and incubated for 48 hours. NO release was determined colorimetrically by Griess reaction. Data are presented as mean ± SEM; * $p<0.0001$ vs. untreated control (u.c.); n = 4.

LPS as positive control induced a NO release of 1369 % ± 64 compared to untreated control. Aqueous myrrh extract in concentrations up to 100 µg/ml did not influence basal NO release compared to untreated control. However, the highest concentration led to an increase in NO release (Figure 47; 0.01 µg/ml MYA: 117.6±5.4; 0.1 mg/ml MYA: 116.6±3.0; 1 µg/ml MYA: 107.7±2.7; 10 µg/ml MYA: 118.4±4.7; 100 mg/ml MYA: 143.5±15.7; 1000 µg/ml MYA: 417.7±76.05, $p<0.0001$; in % untreated control).

3.2.3 Anti-inflammatory activity of coffee charcoal

Effect of coffee charcoal extract on TNBS-induced morphological damage

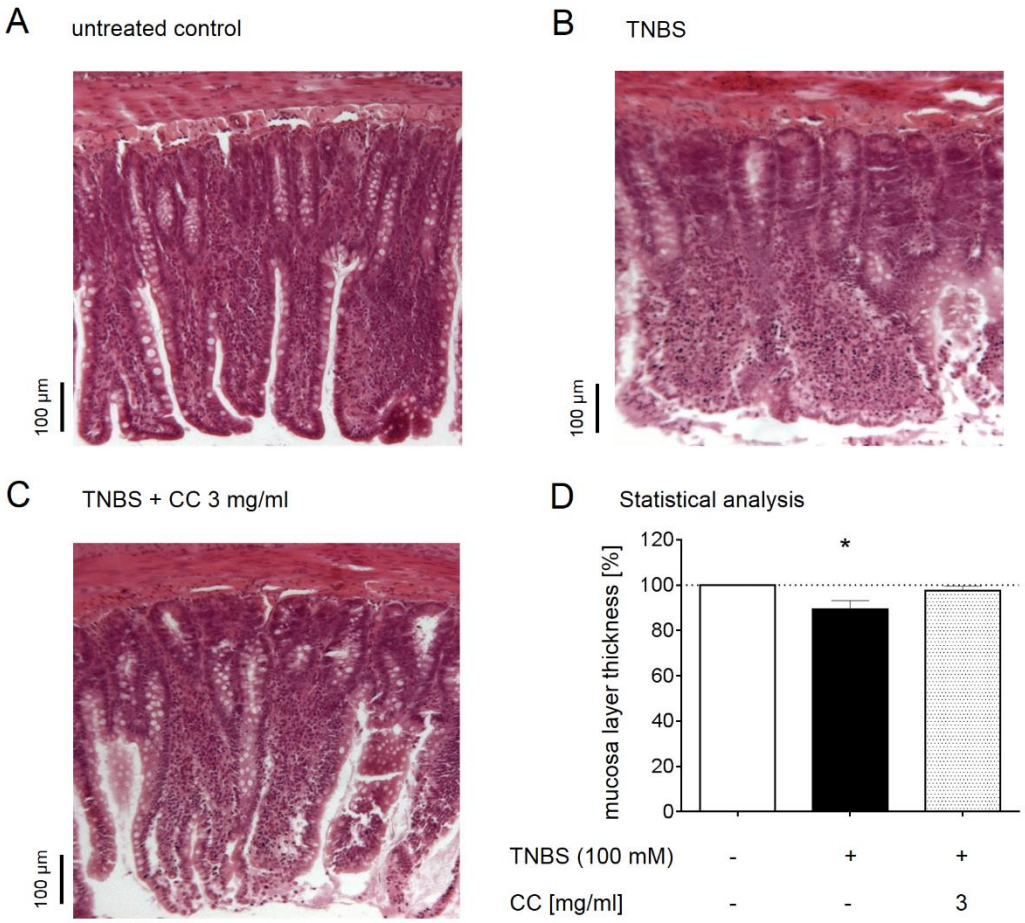

Figure 48: Effect of coffee charcoal extract on TNBS-induced morphological damage. Rat ileum preparations were incubated with TNBS (100 mM) and coffee charcoal extract (CC; 3 mg/ml) for 30 minutes and mucosa layer thickness was determined morphometrically after HE-staining. Single TNBS-incubation served as positive control. Presented are representative original images (A-C) and statistical analysis (D) with mean ± SEM; * $p<0.05$ vs. untreated control (u.c.); n = 4.

Incubation with TNBS (100 mM; 30 minutes) induced a morphological damage expressed by a decrease in mucosa layer thickness down to 89.27 % ± 3.9 compared to untreated control ($p<0.05$). Simultaneous incubation of coffee charcoal extract (CC, 3 mg/ml) did not decrease mucosa layer thickness compared to untreated control (Figure 48; 3 mg/ml CC: 97.65 % ± 2.0; in % untreated control). However, the difference in mucosa layer thickness after single TNBS treatment and simultaneous incubation with coffee charcoal extract (3 mg/ml) was not statistically significant.

Effect of coffee charcoal extract on TNBS-induced contractility decrease

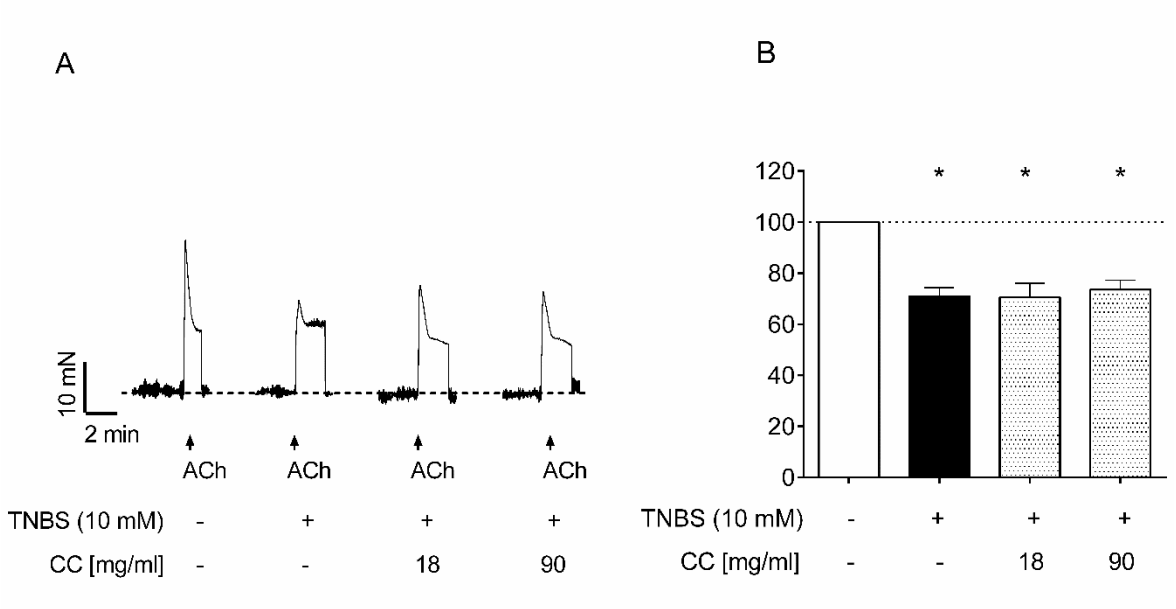

Figure 49: Effect of coffee charcoal extract on TNBS-induced contractility decrease (A: representative original recording; B: statistical analysis).
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and coffee charcoal extract (CC; 17.8 and 89.4 mg/ml) for 30 minutes and ACh-induced contractions were determined using isometric tension measurement. Single TNBS-incubation served as positive control. Presented is a representative original recording (A) and statistical analysis (B) with mean ± SEM; * $p<0.0001$ vs. untreated control (u.c.); n = 6.

Incubation with TNBS (10 mM; 30 minutes) induced a decrease in ACh-induced contraction down to 70.95 % ± 3.4 compared to untreated control ($p<0.0001$). Simultaneous incubation of coffee charcoal extract (CC; 18 and 90 µg/ml) induced a similar effect (Figure 49; 18 µg/ml CC: 70.55±5.5; 90 µg/ml CC: 73.56±3.7; in % untreated control) and had therefore, no effect on TNBS-induced contractility decrease.

Effect of coffee charcoal extract on TNBS-induced TNFα gene expression

The influence of coffee charcoal extract on TNBS-induced overexpression of TNFα-mRNA in rat small intestinal preparations is presented in Figure 50.

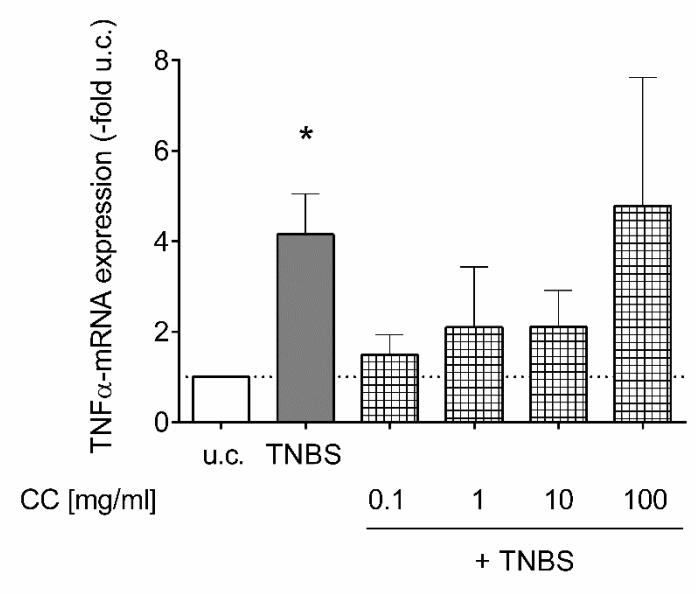

Figure 50: Effect of coffee charcoal extract on TNBS-induced TNFα gene expression
Rat ileum/jejunum preparations were incubated with TNBS (10 mM) and coffee charcoal extract (CC; 0.1 - 100 mg/ml) for 3 hours and the amount of TNFα-mRNA expression was analysed using real time PCR. TNBS-incubation alone served as positive control. Presented are mean ± SEM (n = 5) of TNFα-mRNA expression in relation to untreated control; * $p<0.01$ vs. untreated control.

Incubation with TNBS (10 mM; 3 hours) induced a 4-fold increase in TNFα-mRNA expression compared to untreated control ($p<0.01$). Simultaneous incubation of TNBS and coffee charcoal extract (0.1 – 100 mg/ml) did not influence the mRNA-expression levels of TNFα compared to sole TNBS-stimulation.

Effect of coffee charcoal extract on LPS-induced TNFα release

To determine the influence of coffee charcoal extract on LPS-induced TNFα release, LPS-stimulated (100 ng/ml), differentiated THP-1cells were incubated with coffee charcoal extract (CC, 0.1 – 1500 µg/ml) for four hours. Subsequently, TNFα amount in cell supernatant was determined by ELISA. Differentiated THP-1 cells without LPS stimulation (u.c.) differentiated cells that were solely stimulated with LPS (LPS; 100 ng/ml) and differentiated cells that were treated with LPS and the anti-inflammatory glucocorticoid budesonide (Bud; 1 nM) were additionally tested as controls. TNFα release of LPS-stimulated THP-1 cells was set 100 %.

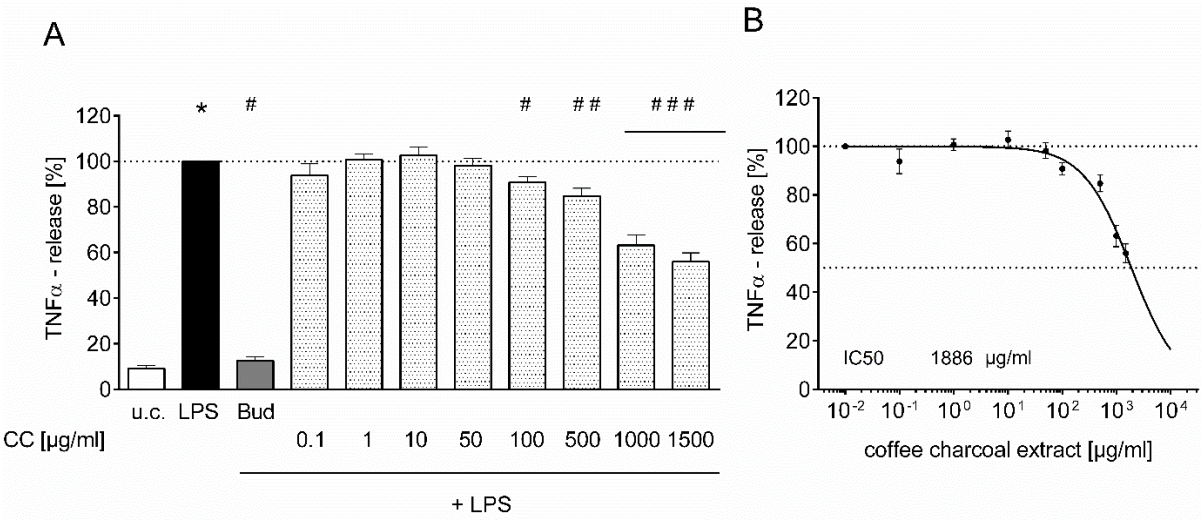

Figure 51: Effect of coffee charcoal extract on LPS-induced TNFα release from THP-1 cells. Differentiated THP-1 cells were treated with LPS (100 ng/ml) and coffee charcoal extract (CC; 0.1 – 1500 µg/ml) and incubated for four hours. Budesonide (Bud, 1 nM) served as positive control. Presented are statistical analysis [A] and concentration-response curve [B]. Data are presented as mean ± SEM and non-linear regression; * $p<0.05$ vs. untreated control (u.c.); # $p<0.05$ vs. LPS, # # $p<0.01$ vs. LPS, # # # $p<0.001$ vs. LPS; n = 8-10.

The influence of coffee charcoal extract (CC, 0.1 – 1000 µg/ml) on LPS-induced TNFα release from THP-1 cells is presented in Figure 51. LPS (100 ng/ml) significantly increased TNFα release in differentiated THP-1 cells (Figure 51; u.c.: 4.35 % ± 1.3; LPS: 100 %, $p<0.0001$). After simultaneous treatment with budesonide (1 nM) TNFα release was significantly decreased compared to single LPS stimulation (Figure 51; LPS: 100 %, Bud: 12.5 % ± 1.7; $p<0.0001$).

Coffee charcoal extract led to a concentration-dependent decrease in TNFα release down to 28 % ± 8.7 with a half maximal inhibitory concentration (IC50) of 1886 µg/ml (Figure 51; 0.1 µg/ml CC: 93.82±5.0; 1.0 µg/ml CC: 100.7±2.4; 10 µg/ml CC: 102.7±3.6; 50 µg/ml CC: 98.19±3.2; 100 µg/ml CC: 90.76±2.6, $p<0.05$; 500 µg/ml 84.77±3.5, $p<0.01$; 1000 µg/ml CC: 63.15±4.4, $p<0.0001$; 1500 µg/ml CC: 56.03±3.8, $p<0.0001$; in % LPS-treatment).

To evaluate the viability of the cells used for determination of TNFα release, MTT-assay and LDH-assay were performed after treatment with the respective substances (Figure 52).

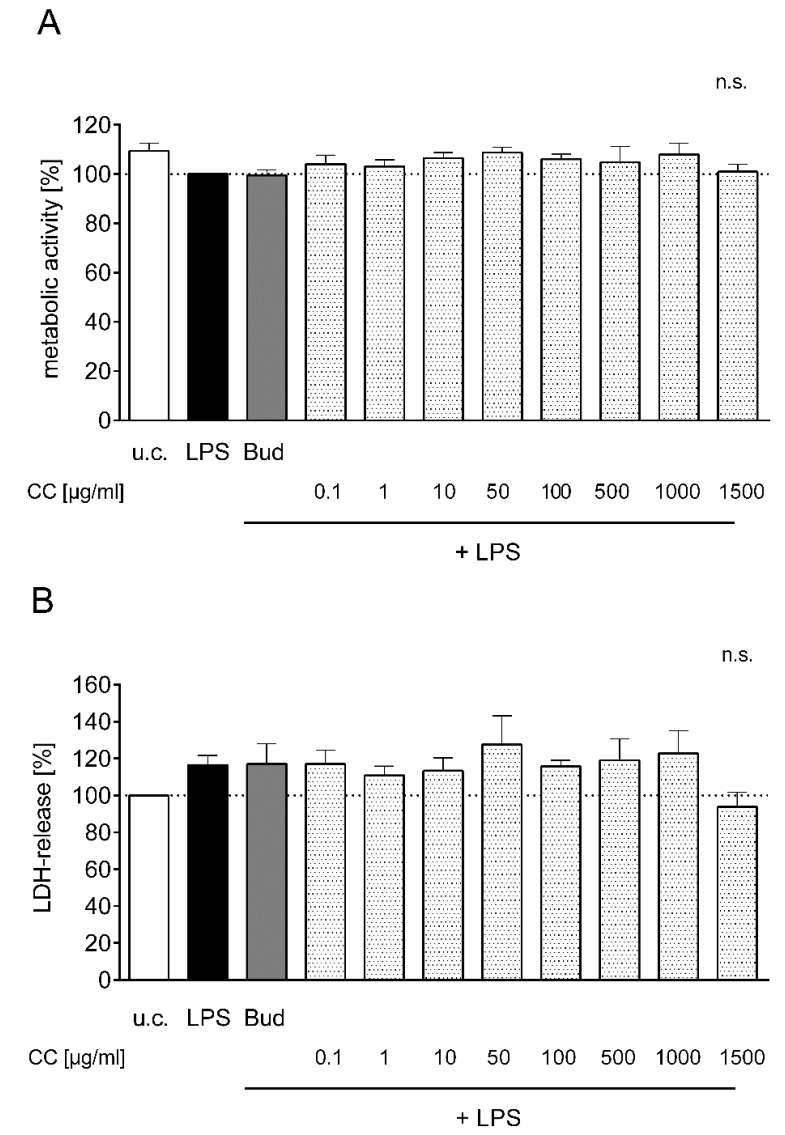

Figure 52: Effect of coffee charcoal extract on metabolic activity (A) and LDH-release (B) of THP-1 cells.
Differentiated THP-1 cells were treated with LPS (100 ng/ml) and coffee charcoal extract (CC; 0.1 – 1500 µg/ml) and incubated for four hours. Metabolic activity was determined by MTT-assay; LDH-assay was performed to determine LDH-release. Untreated control (u.c.); LPS and budesonide (Bud 1 nM) were tested as controls. Data are presented as mean ± SEM (n = 8-10).

Cell viability expressed as metabolic activity of differentiated THP-1 cells was not influenced after simultaneous incubation with LPS (100 ng/ml) and budesonide (1 nM) or coffee charcoal extract in the concentrations tested. Treatment with LPS (100 ng/ml) or simultaneous treatment with budesonide (1 nM) or coffee charcoal extract (0.1–1500 µg/ml) did not alter LDH-release compared to untreated control.

3.3 Microarray gene expression analysis

To investigate the influence of all plant extracts on gene expression profile of human M1-macrophages, LPS/IFNγ-stimulated cells were incubated with the plant extract in selected concentrations. After isolation of the total RNA, gene expression analysis was performed at the Core Unit DNA Technologies of the Interdisciplinary Centre for Clinical Research (IZKF, Medical Faculty, University of Leipzig) using a HumanHT-12 v4 Expression BeadChip.

3.3.1 Effect of LPS/IFNγ-stimulation on gene expression profile of human M1-macrophages

Microarray gene expression analysis was performed to assess alterations in gene expression profile of human M1-macrophages. LPS (100 ng/ml) and IFNγ (10 ng/ml) were used as inflammatory stimulus to activate the macrophages.

Table 25 and Table 26 present an overview of the number of genes that were affected by the LPS/IFNγ-stimulation.

Table 25: Influence of LPS/IFNγ-stimulation on number of regulated genes compared to untreated control (u.c.) (data from first gene expression analysis procedure, 20/03/2014).

Signal LPS/IFNγ / Signal u.c.	Number of regulated genes
> 1000	2
> 100	35
> 10	640
< 0.1	812
< 0.01	25
< 0.001	0

Table 26: Influence of LPS/IFNγ-stimulation on number of regulated genes compared to untreated control (u.c.) (data from second gene expression analysis procedure, 17/04/2014).

Signal LPS/IFNγ / Signal u.c.	Number of regulated genes
> 1000	9
> 100	50
> 10	548
< 0.1	431
< 0.01	19
< 0.001	0

All genes that were up-regulated more than 10-fold were analysed by enrichment analysis. Figure 53 - 54 show a visualisation of the gene ontological enrichment analysis.

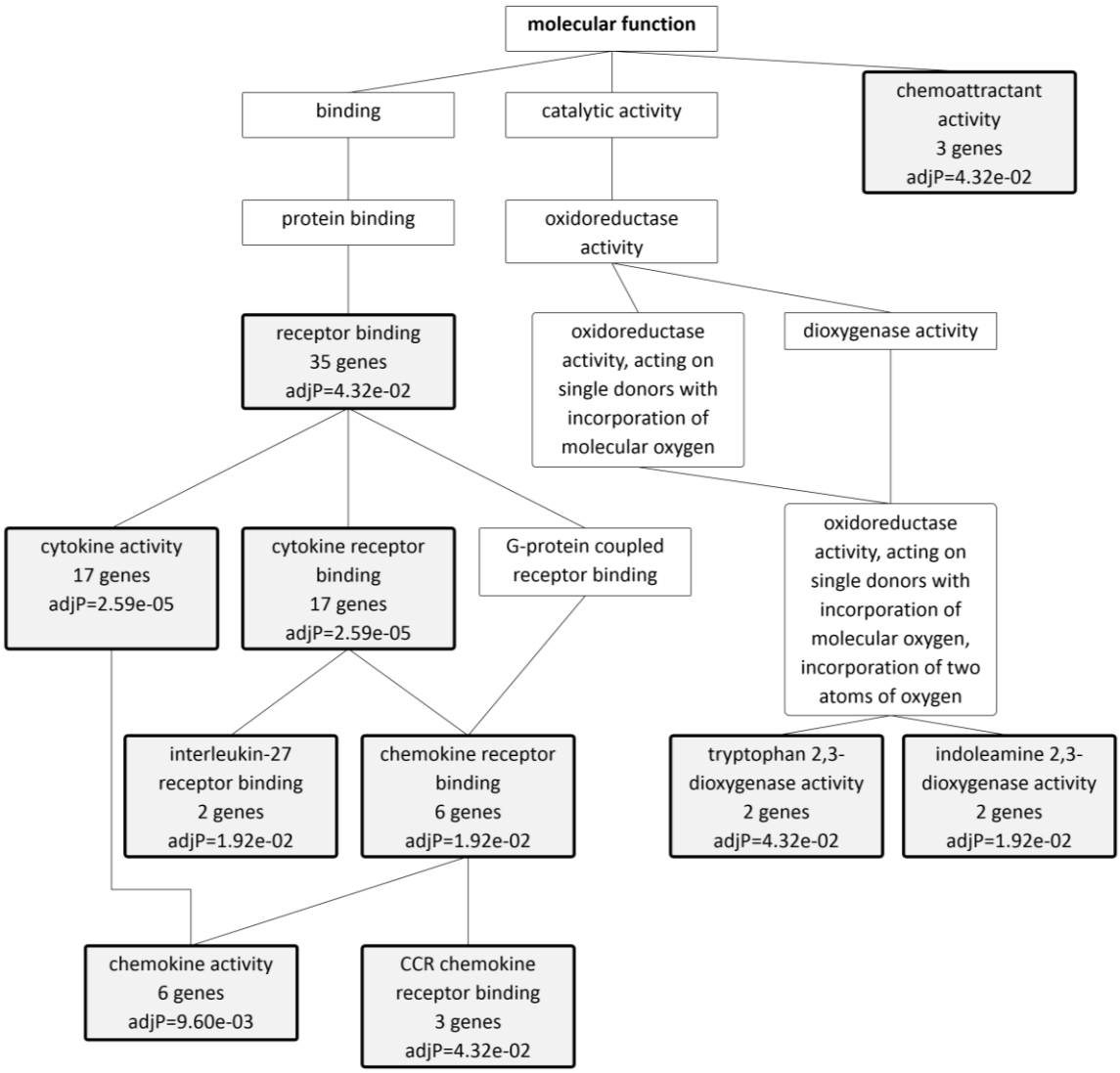

Figure 53: Gene ontological enrichment analysis of gene up-regulation after LPS/IFNγ-stimulation (data from first gene expression analysis procedure, 20/03/2014).
Enrichment analysis of genes that were up-regulated after LPS/IFNγ-stimulation (regulation-factor ≥ 10; total: 640 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values, adjP) are highlighted in grey.

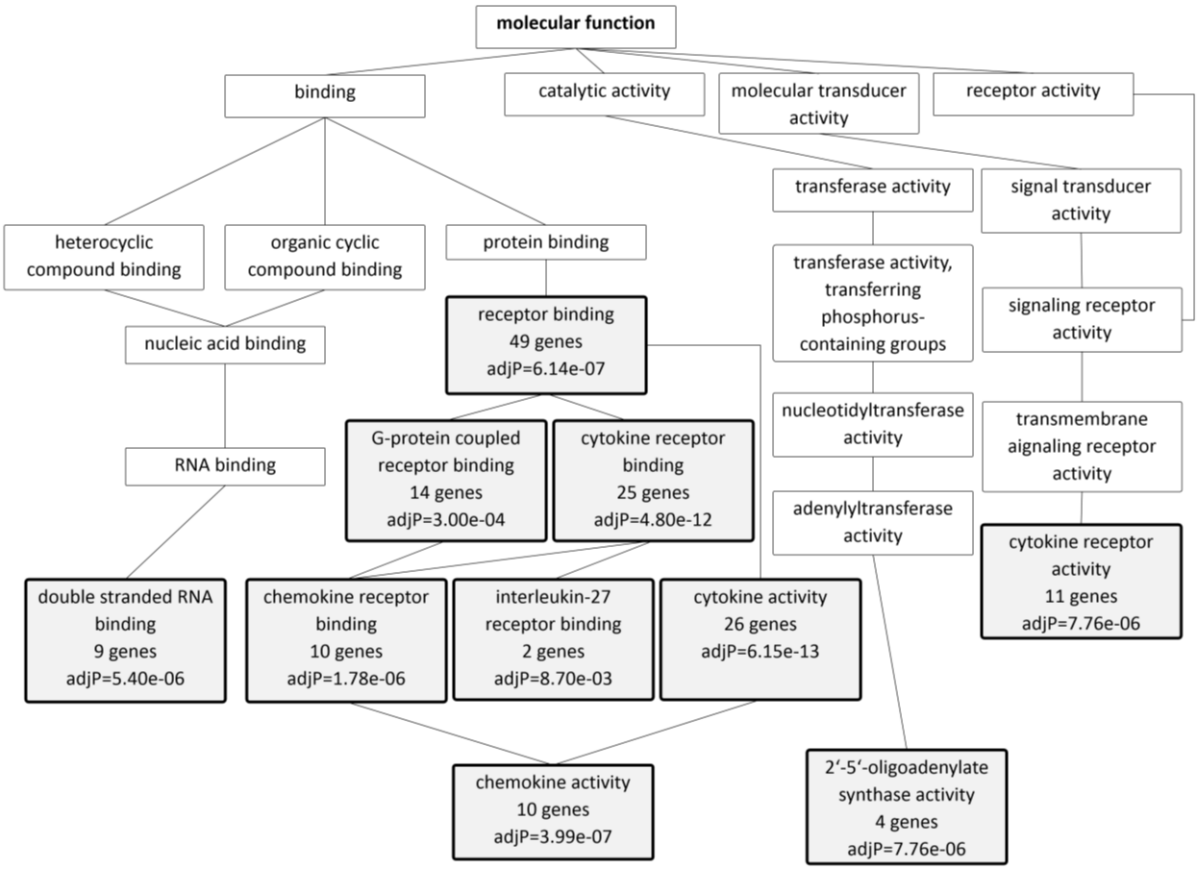

Figure 54: Gene ontological enrichment analysis of gene up-regulation after LPS/IFNγ-stimulation (data from second gene expression analysis procedure, 17/04/2014).
Enrichment analysis of genes that were up-regulated after LPS/IFNγ-stimulation (regulation-factor ≥ 10; total: 548 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Genes that were up-regulated after LPS/IFNγ-stimulation can be classified in GO-categories that are associated with the adaptive immune system like chemokine activity, chemoattractant activity, chemokine activity and the kynurenine pathway of tryptophan metabolism including indoleamine 2,3-dioxygenase activity and tryptophan 2,3-dioxygenase activity.

All genes that were down-regulated more than 10-fold were likewise analysed by enrichment analysis. Figure 55 - 56 show a visualisation of the gene ontological enrichment analysis.

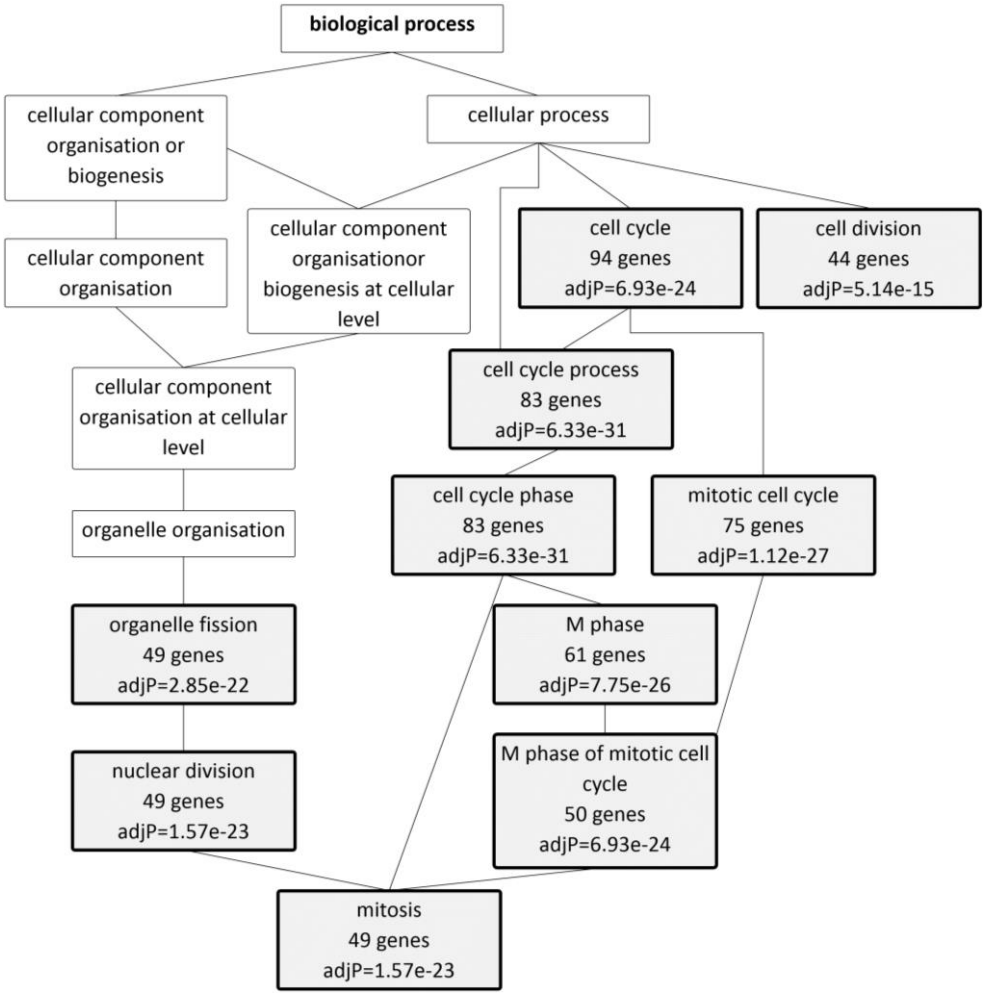

Figure 55: Gene ontological enrichment analysis of gene down-regulation after LPS/IFNγ-stimulation (data from first gene expression analysis procedure, 20/03/2014).
Enrichment analysis of genes that were down-regulated after LPS/IFNγ-stimulation (regulation-factor ≤ 0.1; total: 812 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

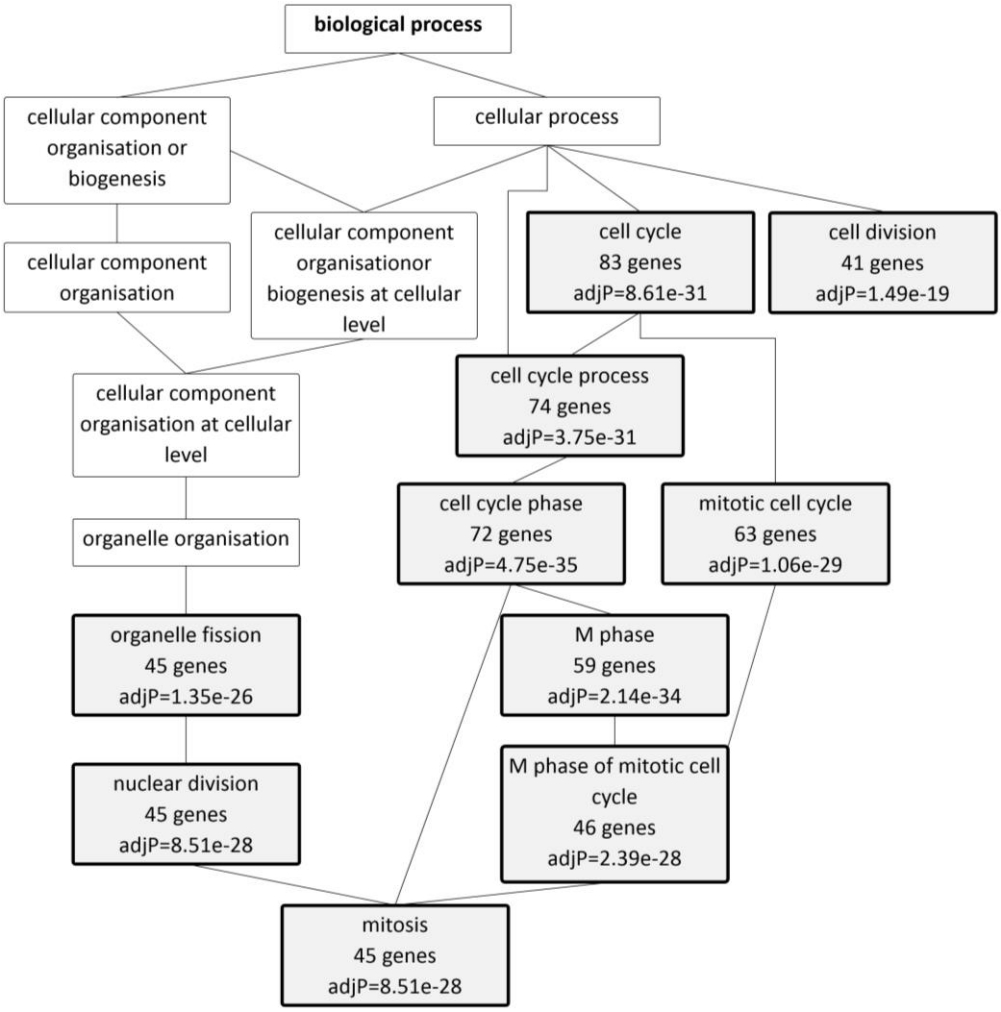

Figure 56: Gene ontological enrichment analysis of gene down-regulation after LPS/IFNγ-stimulation (data from second gene expression analysis procedure, 17/04/2014).
Enrichment analysis of genes that were down-regulated after LPS/IFNγ-stimulation (regulation-factor ≤ 0.1; total: 431 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

GO-categories that were significantly enriched after LPS/IFNγ-stimulation include biological processes associated with cell cycle regulation including cell cycle division and mitosis.

3.3.2 Effect of chamomile flower extract on LPS/IFNγ-induced change in gene expression profile of human M1-macrophages

To assess the influence of chamomile flower on LPS/IFNγ-induced changes in gene expression of human M1-macrophages, cells were treated simultaneously with chamomile flower extract (200 mg/ml) and LPS/IFNγ. Single treatment with LPS/IFNγ and untreated cells served as control.

Table 27 presents an overview of the number of genes that were affected by the treatment with chamomile flower extract compared to LPS/IFNγ-stimulation.

Table 27: Influence of chamomile flower extract (KA) on number of regulated genes compared to LPS/IFNγ-stimulation (data from second gene expression analysis procedure, 17/04/2014).

Signal KA / Signal LPS/IFNγ	Number of regulated genes
> 1000	0
> 100	0
> 10	78
< 0.1	104
< 0.01	0
< 0.001	0

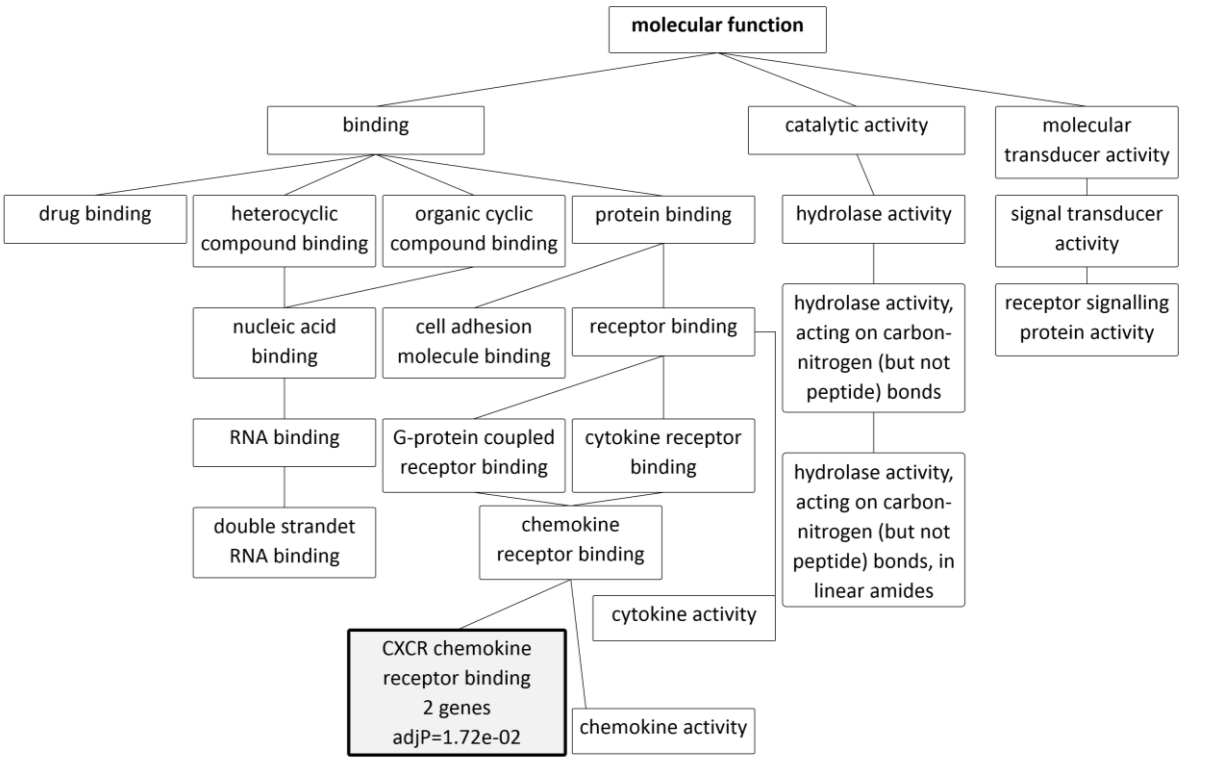

Figure 57: Gene ontological enrichment analysis – Influence of chamomile flower extract (200 mg/ml) on LPS/IFNγ-stimulated gene expression (data from second gene expression analysis procedure, 17/04/2014).
Enrichment analysis of genes that were down-regulated after simultaneous treatment with LPS/IFNγ and chamomile flower extract (200 µg/ml) compared to single LPS/IFNγ-stimulation (regulation-factor ≤ 0.1 vs. LPS/IFNγ-stimulation; total: 104 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Figure 57 presents a visualisation of the gene ontological enrichment analysis. CXCR chemokine receptor binding[1] was the only category that shows a significant enrichment with genes down-regulated more than 10-fold compared to LPS/IFNγ-stimulation.

Genes that were up-regulated after simultaneous treatment with chamomile flower extract compared to LPS/IFNγ-stimulation could not be clustered significantly in GO-categories (adjP ≥ 0.05) (data not presented).

[1] genes concerned: CXCL12 - chemokine (C-X-C Motif) ligand 12; CXCL 13 - chemokine (C-X-C Motif) ligand 13

3.3.3 Effect of myrrh on LPS/IFNγ-induced change in gene expression profile of human M1-macrophages

The influence of ethanolic and aqueous myrrh extract on LPS/IFNγ-induced changes in gene expression of human M1-macrophages was assessed after simultaneous treatment with ethanolic (30 mg/ml) or aqueous (500 µg/ml) myrrh extract and LPS/IFNγ. Single treatment with LPS/IFNγ and untreated cells served as control.

Effect of ethanolic myrrh extract on LPS/IFNγ-induced change in gene expression profile of human M1-macrophages

Table 28 presents an overview of the number of genes that were affected by the treatment with ethanolic myrrh extract compared to LPS/IFNγ-stimulation.

Table 28: Influence of ethanolic myrrh extract (MY) on number of regulated genes compared to LPS/IFNγ-stimulation (data from first gene expression analysis procedure, 20/03/2014).

Signal MY / Signal LPS/IFNγ	Number of regulated genes
> 1000	0
> 100	0
> 10	84
< 0.1	202
< 0.01	0
< 0.001	0

Figure 58 presents a visualisation of the gene ontological enrichment analysis.

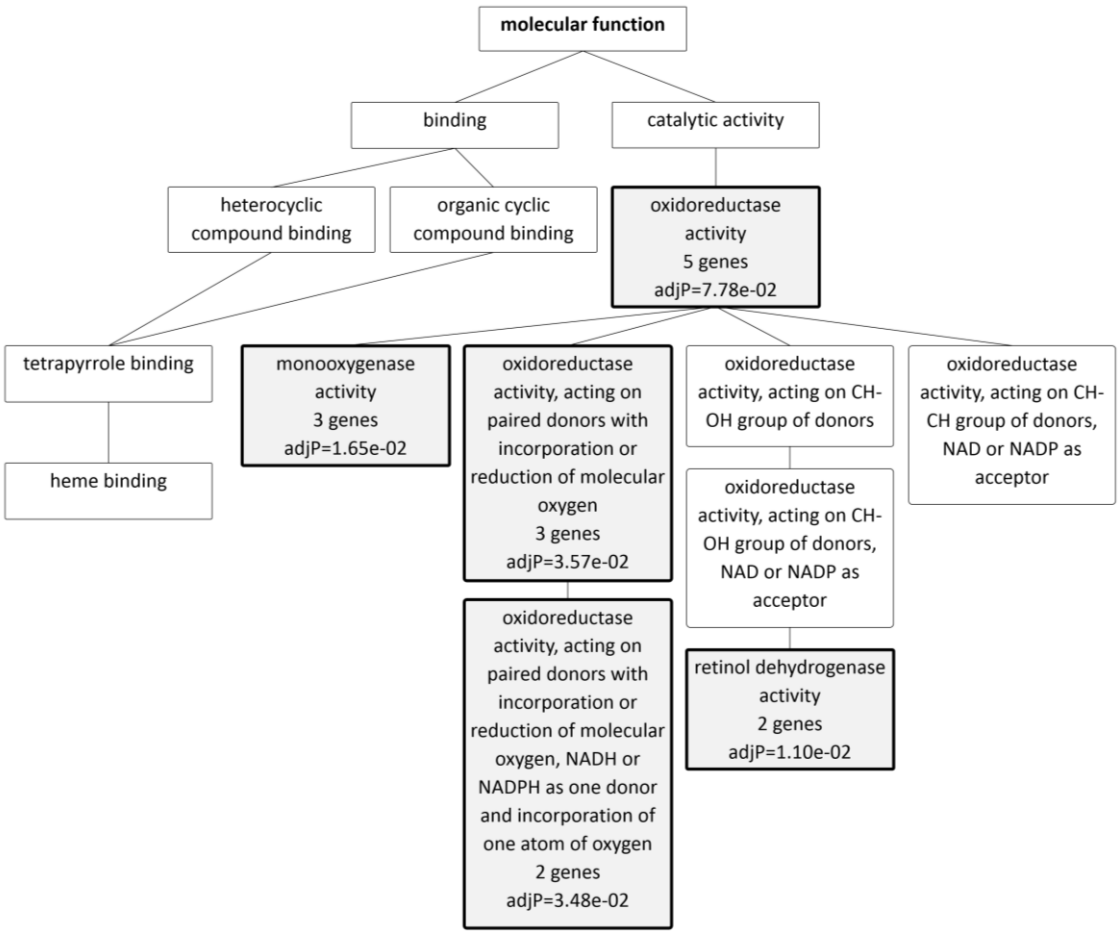

Figure 58: Gene ontological enrichment analysis – Influence of ethanolic myrrh extract (30 mg/ml) on LPS/IFNγ-stimulated gene expression (data from first gene expression analysis procedure, 20/03/2014).
Enrichment analysis of genes that were up-regulated after simultaneous treatment with LPS/IFNγ and ethanolic myrrh extract (30 mg/ml) compared to single LPS/IFNγ-stimulation (regulation-factor ≥ 10 vs. LPS/IFNγ-stimulation; total: 84 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Genes that were up-regulated more than 10-fold compared to LPS/IFNγ-stimulation can be clustered in categories with a significant enrichment, including retinol dehydrogenase activity[2], monooxygenase activity[3] and oxidoreductase activity[4].

Genes that were down-regulated after simultaneous treatment with ethanolic myrrh extract compared to LPS/IFNγ-stimulation could not be clustered in significantly enriched GO-categories (adjP ≥ 0.05) (data not presented).

[2] genes concerned: SDR9C7 - short chain dehydrogenase/reductase family 9C, member 7; AKR1C3 - aldo-keto reductase family 1, member C3
[3] genes concerned: CYP2U1 - cytochrome P450, family 2, subfamily U, polypeptide 1; AKR1C3 - aldo-keto reductase family 1, member C3; CYP4F3 - cytochrome P450, family 4, subfamily F, polypeptide 3
[4] genes concerned: AKR1C3 - aldo-keto reductase family 1, member C3; CYP4F3 - cytochrome P450, family 4, subfamily F, polypeptide 3

Effect of aqueous myrrh extract on LPS/IFNγ-induced change in gene expression profile of human M1-macrophages

Table 29 presents an overview of the number of genes that were affected by the treatment with aqueous myrrh extract compared to LPS/IFNγ-stimulation.

Table 29: Influence of aqueous myrrh extract (MYA) on number of regulated genes compared to LPS/IFNγ-stimulation (data from second gene expression analysis procedure, 17/04/2014).

Signal MYA / Signal LPS/IFNγ	Number of regulated genes
> 1000	0
> 100	0
> 10	25
< 0.1	112
< 0.01	0
< 0.001	0

Genes that were up- or down-regulated after simultaneous treatment with aqueous myrrh extract compared to LPS/IFNγ-stimulation by the factor 10 could not be clustered in significantly enriched GO-categories (adjP ≥ 0.05) (data not presented).

3.3.4 Effect of coffee charcoal extract on LPS/IFNγ-induced change in gene expression profile of human M1-macrophages

The influence of coffee charcoal extract on LPS/IFNγ-induced changes in gene expression of human M1-macrophages was assessed after simultaneous treatment with coffee charcoal extract and LPS/IFNγ. Single treatment with LPS/IFNγ and untreated cells served as control.

Table 30: Influence of coffee charcoal extract (CC) on number of regulated genes compared to LPS/IFNγ-stimulation (data from first gene expression analysis procedure, 20/03/2014).

Signal CC / Signal LPS/IFNγ	Number of regulated genes
> 1000	**1**
> 100	**5**
> 10	**195**
< 0.1	**372**
< 0.01	**6**
< 0.001	**0**

Figure 59 - 60 present a visualisation of the gene ontological enrichment analysis.

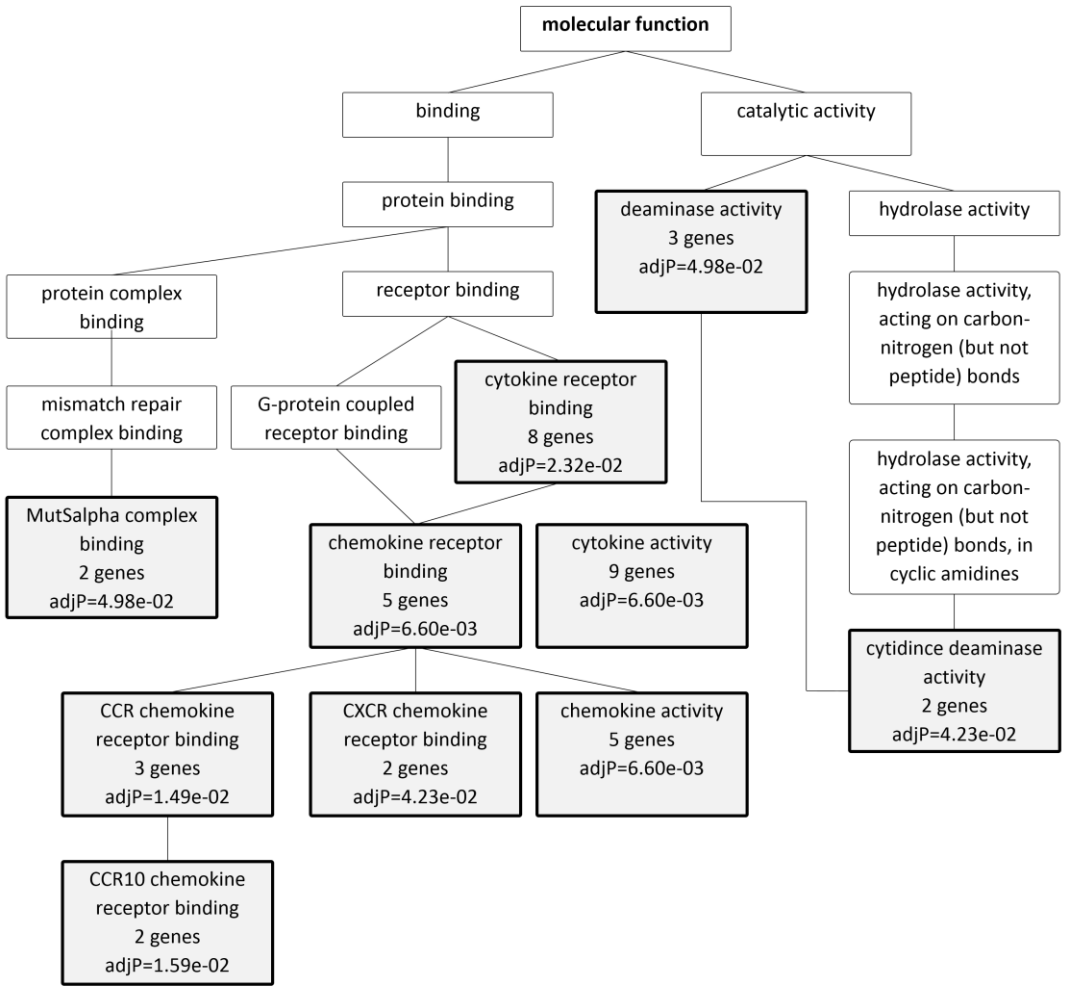

Figure 59: Gene ontological enrichment analysis – Influence of coffee charcoal extract (500 mg/ml) on LPS/IFNγ-stimulated gene expression (data from second gene expression analysis procedure, 20/03/2014).
Enrichment analysis of genes that were down-regulated after simultaneous treatment with LPS/IFNγ and charcoal extract (500 mg/ml) compared to single LPS/IFNγ-stimulation (regulation-factor ≤ 0.1 vs. LPS/IFNγ-stimulation; total: 372 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Genes that were down-regulated more than 10-fold compared to LPS/IFNγ-stimulation can be clustered in significantly enriched categories associated with chemokine signalling including chemokine activity, CXCR chemokine receptor binding and CCR chemokine receptor binding (Figure 59).

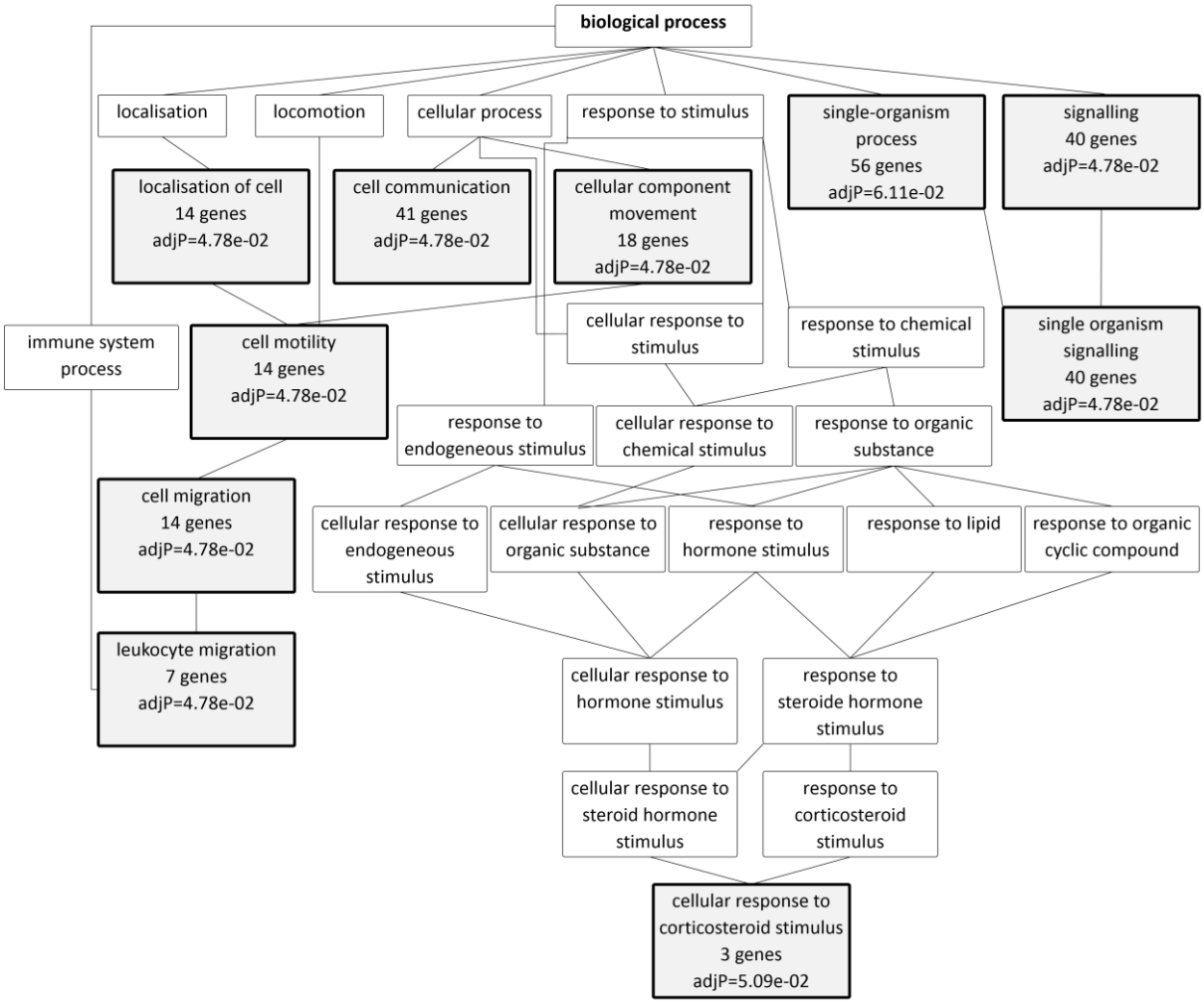

Figure 60: Gene ontological enrichment analysis – Influence of coffee charcoal extract (500 mg/ml) on LPS/IFNγ-stimulated gene expression (data from second gene expression analysis procedure, 20/03/2014).
Enrichment analysis of genes that were up-regulated after simultaneous treatment with LPS/IFNγ and charcoal extract (500 mg/ml) compared to single LPS/IFNγ-stimulation (regulation-factor ≥ 10 vs. LPS/IFNγ-stimulation; total: 195 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Genes that were up-regulated more than 10-fold compared to LPS/IFNγ-stimulation can be clustered in significantly enriched categories associated with cell motility such as cell migration and leukocyte migration and cellular response to corticosteroid stimulus (Figure 60).

3.3.5 Effect of aqueous myrrh extract on gene expression profile of human M1-macrophages

The influence of aqueous myrrh extract (500 µg/ml) on gene expression of human M1-macrophages was assessed compared to untreated cells (Table 31).

Table 31: Influence of aqueous myrrh extract (MYA) on number of regulated genes compared to untreated control (u.c.) (data from second gene expression analysis procedure, 17/04/2014).

Signal MYA / Signal u.c.	Number of regulated genes
> 1000	1
> 100	20
> 10	678
< 0.1	255
< 0.01	0
< 0.001	0

Figure 61 presents a visualisation of the gene ontological enrichment analysis.

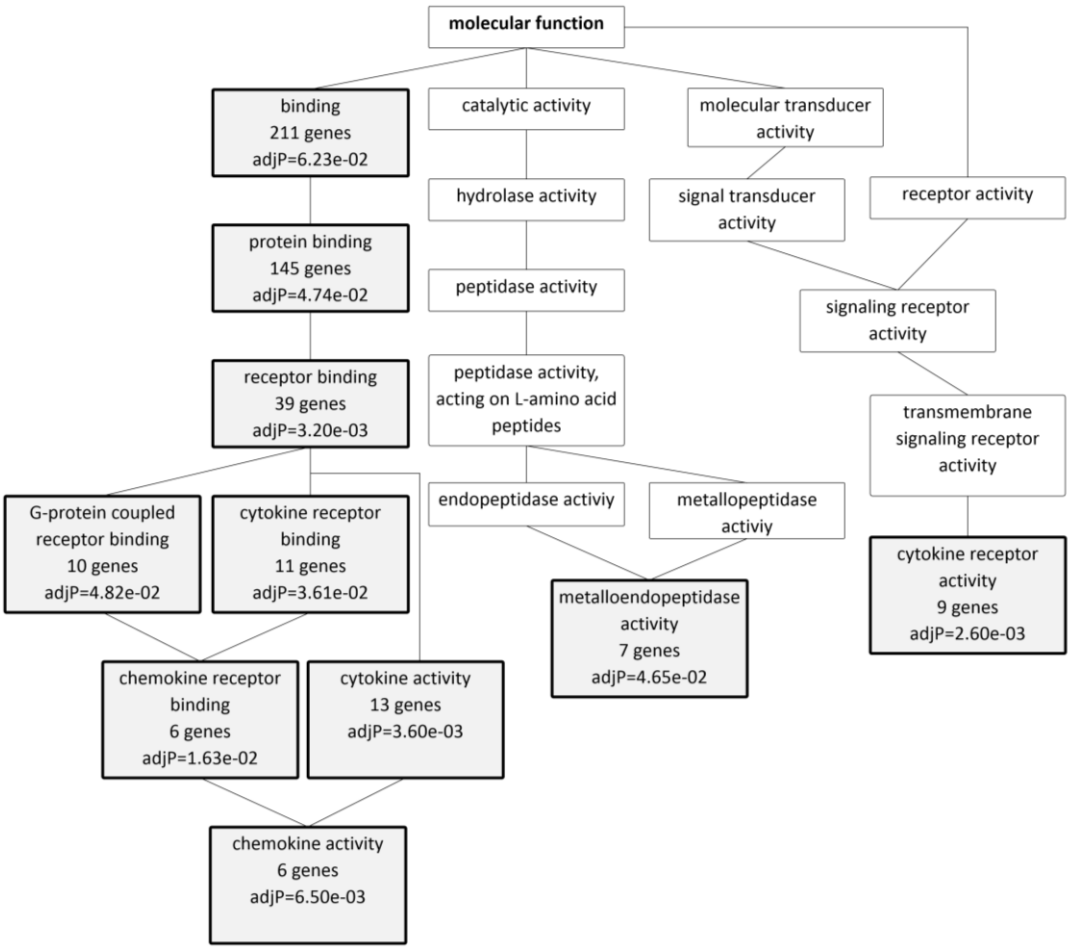

Figure 61: Gene ontological enrichment analysis of gene up-regulation after single treatment with aqueous myrrh extract (500 mg/ml)
Enrichment analysis of genes that were up-regulated after single treatment with aqueous myrrh extract (500 mg/ml) (regulation-factor ≥ 10; total: 678 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Genes that were up-regulated more than 10-fold compared to untreated control can be clustered in significantly enriched categories associated with chemokine signalling including chemokine and cytokine activity (Figure 61).

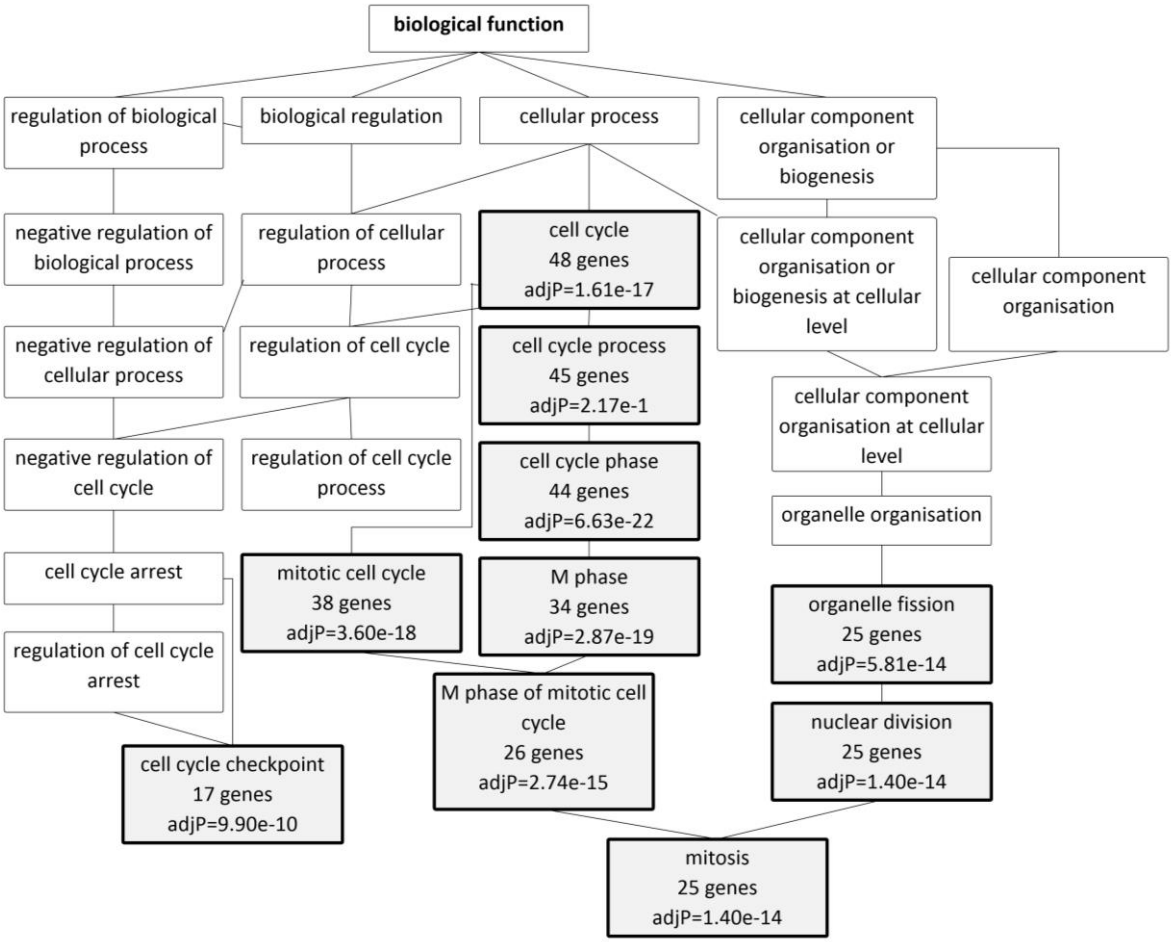

Figure 62: Gene ontological enrichment analysis of gene down-regulation after single treatment with aqueous myrrh extract (500 mg/ml)
Enrichment analysis of genes that were down-regulated after single treatment with aqueous myrrh extract (500 mg/ml) (regulation-factor ≥ 10; total: 255 genes). Analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt software, version 1/30/2013). Presented are categories including two or more genes; hypergeometric test with Multiple Test Adjustment BH; categories with the ten lowest significance levels (adjusted p-values) are highlighted in grey.

Genes that were down-regulated more than 10-fold after treatment with aqueous myrrh extract compared to untreated control can be clustered in significantly enriched categories associated with cell cycle regulation such as cell cycle checkpoint, nuclear division and mitosis (Figure 62).

4 Discussion

4.1 Chamomile flower

4.1.1 Extract characteristics

Varying quality and origin of the plant material as well as different extraction methods result in significant differences in pharmaceutical quality and hence therapeutic effect of chamomile preparations. The place of cultivation determines for example the composition of the essential oil whereby bisabololoxid-A or –B rich strains as well as varying matricin content can be found. The extraction solvent as well as extraction method determines the composition of the finished extract. In contrast to pure aqueous extracts, which contain only water soluble flavonoids and mucilages, ethanolic-aqueous extracts contain polar (flavonoids) as well as apolar (essential oil) components (Schilcher 2004). The essential oil content is determined by the ethanol-water ratio, whereby higher ethanol concentrations e.g. 70 % (v/v) lead to an essential oil concentration of 0.25 %, in contrast to an ethanol concentration of 40 % (v/v), which leads to 0.10 % essential oil concentration (Münzel and Huber 1961).

In the fixed combination Myrrhinil-Intest®, a dried chamomile flower extract is processed. The dried extract was obtained after ethanolic (60 % m/m) extraction of chamomile flowers with a drug-extract-ratio of 4-6 to 1.

4.1.2 Spasmolytic activity of the chamomile flower extract

The antispasmodic effects of chamomile flower have been demonstrated in several animal models (Hava and Janku 1957; Achterrath-Tuckermann *et al.* 1980; Forster *et al.* 1980).

Purpose of the present study was to characterise the spasmolytic activity of the chamomile flower extract used in Myrrhinil-Intest® and to define an effective concentration range using a well-established *in vitro* model (Michael 2008). Therefore, the extract was tested in different concentrations, using isometric tension measurement in isolated rat small intestinal preparations.

The examined chamomile flower extract inhibited the ACh-induced contractions concentration-dependently. The highest concentration tested decreased the contractions down to 56 %. In this experiment setting, a half maximal inhibitory concentration of 160.7 µg/ml was determined.

These findings are in accordance with previous studies, where the spasmolytic activity of chamomile flower extract and components thereof was examined. Forster and co-workers reported that an ethanolic chamomile flower extract (31 % v/v; DER: 1:3.5) shifted the concentration-response curve of the spasmogenic substances acetylcholine and histamine to

the right in a dose-dependent manner (Forster *et al.* 1980). Investigating its inhibitory effect on ACh-induced contractions of guinea-pig ileum, Achterrath-Tuckermann *et al.* (1980) found that total chamomile flower extract (38.5 % v/v; DER: 1:4-4.5) effectively inhibited the spasmogenic actions of barium chloride, histamine, acetylcholine, serotonin and bradykinin. Responsible for the spasmolytic activity are flavonoids (apigenin > quercetin > luteolin > kaempferol) and their glycosides, but also the lipophilic compounds of the essential oil like bisabolol and bisabololoxide A and B (Achterrath-Tuckermann *et al.* 1980).

It was found by Hörhammer and confirmed later, that the spasmolytic effect of the chamomile flower components is mediated directly at the muscle cells (musculotrop) (Hörhammer *et al.* 1963; Achterrath-Tuckermann *et al.* 1980). A possible mechanism of action was provided by Maschi and co-workers, who reported, that inhibition of cAMP-phosphodiesterase (cAMP-PDE) is a potential mechanism responsible for the spasmolytic effects. Inactivation of cAMP-PDE leads to elevated intracellular cAMP levels which cause relaxation in gastrointestinal muscle. Especially after apigenin and luteolin application, the activity of human cAMP-PDE was decreased (Maschi *et al.* 2008).

A clinical study (prospective, randomised, double-blind) by de la Motte's group confirmed the effectiveness of a co-medication with chamomile flower for the treatment of acute diarrhea in children (six month to five years old) (de la Motte *et al.* 1997). These findings are supported by an *in vivo* study by Di Carlo, who reported a reduced intestinal transit time in mice after intraperitoneal administration of the chamomile flavonoid apigenin (12.5 – 50 mg/kg) amongst others (Di Carlo *et al.* 1993).

Another aspect of the present studies was the revision of spasmolytic effects under inflammatory conditions. As previously pointed out, inflammation has been found to play a crucial role in the pathophysiology of functional bowel disorders such as IBS. Persisting inflammatory processes can lead to a number of functional and structural alterations in the concerned tissue impairing contractility and mucosal barrier function. It is well established that inflammation markedly alters motility patterns of the gastrointestinal tract. Phasic and tonic contractions have been shown to be suppressed, whereas the giant migration peristaltic contractions of large amplitude appear to be increased in frequency generating diarrheal symptoms associated with inflammation (Malykhina and Akbarali 2004).

Several studies reported a decreased small intestinal motility after induced inflammation (Moreels *et al.* 2001; Demedts *et al.* 2006; Grossi *et al.* 1993). Since these alterations in motility could impact the spasmolytic activity of chamomile flower extract, isometric tension measurement was repeated after inflammation was induced using an *in vitro* TNBS inflammation model (Warstat 2004). The spasmolytic activity of the examined chamomile

flower extract was comparable in inflamed tissue and similar concentrations induced a half maximal inhibitory effect (IC50 in untreated preparations: 160 µg/ml vs. IC50 in inflamed preparations 126.1 µg/ml).

4.1.3 Anti-inflammatory activity of chamomile flower

The anti-inflammatory activity of chamomile flowers is well established and reported in literature (Della Loggia *et al.* 1990; Tubaro *et al.* 1984; Al-Hindawi *et al.* 1989; Gerritsen *et al.* 1995).

The present study aimed to characterise the anti-inflammatory potential of the chamomile flower extract used in Myrrhinil-Intest®. Based on the indication area, the studies focused on the intestinal tract.

In a first set of experiments, the effect of the chamomile flower extract was examined in a TNBS inflammation model. TNBS is a well-established model substance to induce inflammation in the gastrointestinal tract (Blumberg *et al.* 1999). The advantages and limitations of this *in vitro* model have been discussed earlier. Briefly, it provides the advantage of a fast and cost efficient screening method for anti-inflammatory agents, since it effectively models acute inflammatory processes. However, its limitations as an *in vitro* model lacking an intact cardiovascular system are obvious, since various hormonal and neuronal influences are absent. Nonetheless, the TNBS-induced morphological, immunohistochemical, molecularbiological and functional changes as results of acute inflammatory processes in the *in vitro* model correlate with findings *in vivo* and establish the capability of the *in vitro* model as an alternative to *in vivo* studies (Warstat 2004; Michael *et al.* 2012). It is not surprising that inflammatory processes can be observed in an *in vitro* model although systemic circulation is lacking. The immune system of the gastrointestinal tract, also described as gut associated lymphoid tissue, hosts a high number of cells of the innate and adaptive immune system which mediate inflammation (Miura *et al.* 2011).

In the present studies, the degree of TNBS-induced inflammatory processes was estimated by three different parameters: morphological damage, contractility and TNFα gene expression. TNBS reduced mucosa layer thickness of ileal preparations by about 20 %. This reduction is attributed to necrotic processes as demonstrated earlier by Michael, who performed immunohistochemical studies with Sytox Green (Michael 2008).

The ACh-induced contractions of small intestinal preparations are reduced by about 30 %. The mechanisms behind this decrease in contractility is controversially discussed. Alterations in nonadrenergic-noncholinergic signalling as well as alterations in calcium-mobilisation amongst others seem to contribute to the loss in functionality (Poli *et al.* 2001; Shi and Sarna 2000).

Furthermore, TNBS treatment leads to an increased production of TNFα-mRNA by the factor 4, which confirms the involvement of inflammatory processes. *In vivo* studies confirm an increased TNFα gene expression in inflamed tissue after TNBS treatment (Shi *et al.* 2011; Strober and Fuss 2006).

The anti-inflammatory activity of chamomile flower extract could be confirmed histologically whereby an intermediate concentration (3 mg/ml) inhibited the TNBS-induced reduction of mucosal layer thickness. However, high concentration of chamomile flower extract (30 mg/ml) led to a further decrease in mucosal layer thickness, indicating unspecific opposing effects, which occur in higher concentrations.

These observations could be reinforced in functional studies looking at the TNBS-induced contractility decrease. Intermediate concentrations of the chamomile flower extract (3 and 10 mg/ml) inhibited the TNBS-induced reduction of ACh-induced contractions in small intestinal preparations. After chamomile treatment, the preparations responded to the spasmogenic stimulus ACh to the same extent as the untreated control. The higher concentrations however could not inhibit this decrease in contractility, yet it further reduced the contractile response. An inverted U-shape concentration-response relation is quite commonly seen in the pharmacology of herbal preparations, whereby higher concentrations often induce unspecific effects which can negatively affect physiological processes.

Analysis of the TNFα gene expression revealed that chamomile flower extract suppressed the TNBS-induced overexpression of TNFα in all concentrations tested (0.1–100 mg/ml). This leads to the impression that the gene expression of TNFα is affected by chamomile flower extract in much lower concentrations and involves the suspicion that unspecific effects occur in the concentrations tested. However, this observation supports the assumption that the reported effects on TNBS-induced morphological damage and contractility decrease are induced by anti-inflammatory effects, since TNFα is a major pro-inflammatory mediator and its gene expression is induced in the early stages of inflammation.

To validate the previous results and to get a more precise concentrations response profile a simplified inflammation model was used. In this cell culture model, LPS was used to induce a pro-inflammatory response from human differentiated macrophage like cells. This model allows a more precise investigation of the release of pro-inflammatory TNFα, since one monoclonal cell type can be observed. Thus, a potential interference by heterogeneity of experimental animals and molecular mechanisms of other cell types for example can be excluded.

Chamomile flower extract inhibited the LPS-induced TNFα release from THP-1 cells concentration dependently down to 28 % with an IC50 of 438.8 µg/ml.

Since the occurrence of unspecific effects in higher concentrations is known from previous experiments, cytotoxicity testing was performed to exclude potential interferences with the observed inhibition of TNFα release. LDH release as a measure for necrotic cell death was not influenced by chamomile flower extract. In the highest concentration tested (1000 µg/ml) chamomile flower extract reduced metabolic activity down to 70 %. The resulting IC50 of 3108 µg/ml in relation to the IC50 for TNFα release resulted in a selectivity index of 7.08, which means that a reduction of metabolic activity occurs in 7-fold higher concentrations than the inhibition of TNFα release.

The observed suppression of TNBS-induced gene expression of TNFα and the inhibition of LPS-induced TNFα release could be one possible mechanism mediating the anti-inflammatory effects of chamomile flower extract. Yet, further details about mechanisms of action are known from literature. Chamazulene, α-bisabolol and flavonoids like apigenin possess the highest anti-inflammatory activity against pro-inflammatory agents by inhibiting 5-lipoxygenase and therefore the formation of pro-inflammatory leukotriene B4 (Ammon *et al.* 1996). Furthermore, it was found that an aqueous chamomile flower extract exerts its anti-inflammatory activity via inhibition of cyclooxygenase-2 (COX-2) enzymes (Srivastava *et al.* 2009). Both 5-lipoxygenase and COX-2 are key enzymes in the conversion of arachidonic acid into pro-inflammatory leukotrienes or prostaglandins respectively, which contribute to the regulation of the cytokine expression profile.

Microarray gene expression analysis

Gene expression analysis was performed to assess the influence of chamomile flower extract on the gene expression profile of stimulated human macrophages. Therefore, a model to study activated M1-macrophages was used, which has been characterised earlier (Jaguin *et al.* 2013).

LPS/IFNγ-stimulation triggers an inflammatory response, which can be characterised by an up-regulation of genes associated with innate and adaptive immune system. This was observed in both gene expression analysis experiments. A significant number of genes associated with chemokine signalling with functions including chemoattractant activity, chemokine activity and CCR chemokine receptor binding was up-regulated more than 10-fold after LPS/IFNγ-stimulation according to a gene ontological enrichment analysis (WebGestalt software, version 1/30/2013). Chemokines are important mediators in the early phase of immune response as their main function is to induce chemotaxis of reactive immune cells.

Moreover, a significant number of genes associated with the kynurenine pathway of tryptophan metabolism with functions including indoleamine 2,3-dioxygenase activity and tryptophan 2,3-dioxygenase activity were up-regulated more than 10-fold. Upon immune stimulation and cytokine release, enzymes like indoleamine 2,3-dioxygenase (IDO) are strongly up-regulated to convert systemic tryptophan to N-formylkynurenine. Conversion of tryptophan to kynurenine - as part of the immune defence - is a nearly universal response to IFNγ-release (Moffett and Namboodiri 2003). However, it was found, that a simultaneous stimulation with LPS effectively potentiates IFNγ-induced IDO activity (Hissong and Carlin 1997). The prevailing view is that tryptophan catabolism acts to reduce the amount of tryptophan available to pathogens (‘tryptophan depletion theory’; Moffett and Namboodiri 2003).

On the other hand, a significant number of cells associated with cell cycle regulation were down-regulated more than 10-fold. That appears plausible since monocytes/macrophages undergo various changes in survival and death signalling. Under normal circumstances, monocytes circulate in the bloodstream for a very short time before undergoing spontaneous apoptosis, which is induced by a constitutively active cell death program. Upon differentiation to macrophages and subsequent activation, apoptosis is blocked, sustaining a prolonged survival of monocytes. Macrophages, have acquired mechanisms that inhibit the apoptotic program and activate survival pathways responsible for promoting a longer life span (Parihar *et al.* 2010).

Furthermore it is known, that numerous transcriptional repressors are amongst the repressed genes, which are responsible for regulation and resolution of the inflammatory response (Medzhitov and Horng 2009). Overall, the number and magnitude of expression of induced genes is almost well balanced by the numbers of repressed genes.

To test the influence of chamomile flower extract on the gene expression profile of the macrophages, a concentration of 200 µg/ml was chosen based on previous experiments. This concentration lies in the lower region of the effective concentration range but was considered favourable in light of preliminary flow cytometry experiments with the same cell type.

Treatment with chamomile flower extract (200 mg/ml) did not markedly influence the up- or down-regulation of genes compared to a single LPS/IFNγ-treatment. Only a number of 78 genes were up-regulated more than 10-fold, whereas no genes were up-regulated more than 100-fold compared to LPS/IFNγ. A number of 104 genes were down-regulated more than 10-fold, whereas no genes were down-regulated more than 100-fold.

Of the 104 genes that were down-regulated compared to LPS/IFNγ, two genes could be clustered in a significantly enriched GO-category (CXCR receptor binding). The two genes that

were clustered in this category are the chemokine receptor ligand 12 and 13 (CXCL12 and CXCL13). As indicated earlier, chemokines are important mediators in the early phase of immune response as their main function is to induce chemotaxis of reactive immune cells. The expression of chemokines and their corresponding receptors is triggered by pro-inflammatory stimuli like LPS and IFNγ, as it was observed in this studies. The down-regulation of these genes could hint to a mechanism of the anti-inflammatory activity of chamomile flower. However, it is apparent, that more mechanisms are involved within the complex anti-inflammatory interplay. One reason for the weak manifestation of influenced gene expression could be a too low test concentration.

Taken together with the results from the TNBS- and LPS-inflammation model, the existence of anti-inflammatory effects could be demonstrated for the chamomile flower extract used in Myrrhinil-Intest®.

4.2 Myrrh

4.2.1 Extract characteristics

Myrrhinil-Intest® contains myrrh, whereby the powdered substance of the dried resin and no extracts thereof is processed in the finished product. Therefore it was necessary to create different extracts which contain the spectrum of polar and non-polar ingredients to be investigated. For this purpose, an aqueous and an ethanolic extract was created. The water soluble gum fraction of powdered myrrh is composed of a heterodisperse mixture of proteoglycans with a dominating amount of uranic acid rich compounds (Wiendl *et al.* 1995)

An extract of the ethanol-soluble fraction was provided by DS Pharma. It consists of the ethanol-soluble resin, which is comprised of sesquiterpenes and diterpenic (commiphoric acids) and triterpenic acids as well as their esters (Hanus *et al.* 2005). Due to the water steam distillation process the volatile oil was removed and is therefore not present in the finished ethanolic extract.

4.2.2 Spasmolytic activity of myrrh

The traditional use of myrrh for the treatment of diarrhea has been reported and thereafter been investigated *in vivo* determining antidiarrheal activity in an intestinal loop mice model whereby *Commiphora* species inhibited the toxin induced hypersecretion (Claeson and Samuelsson 1989). Smooth muscle relaxing properties of ethyl acetate myrrh extract fractions have been studied *in vitro* using an isolated guinea pig ileum model. The sesquiterpen T-cadinol seems to be the major active substance inducing muscle relaxing effects (Claeson

et al. 1991a). Based on these findings, the spasmolytic activity of the ethanolic myrrh extract only was investigated.

The results of the present study confirm the postulated muscle relaxing effects. The ACh-induced contractions of rat small intestinal preparations were reduced concentration-dependently down to about 10 % with a half maximal inhibitory concentration of 157.7 µg/ml. Moreover, the basal muscular tone was reduced after application of ethanolic myrrh extract (150 µg/ml).

Further studies using isolated rat aorta and a radioligand binding assay indicated that the muscle relaxing effects could be mediated by calcium antagonistic properties of the sesquiterpene T-cadinol and other terpenic compounds (Claeson *et al.* 1991b; Zygmunt *et al.* 1993; Andersson *et al.* 1997). Therefore, the calcium antagonistic properties of the ethanolic myrrh extract were examined in this study. It was found, that the myrrh-induced decrease of basal muscular tone could be antagonised with the calcium channel agonist Bay K8644. These effects could be reproduced with the well-known calcium channel antagonist nimodipine. An evaluation of the concentration-response relation revealed a significant right-ward shift of the agonistic concentration-response curve, indicating a competitive antagonism. However, these observations were made at comparably low myrrh concentrations (150 µg/ml). To get a complete picture of the pharmacological activity it was of interest to test higher concentrations additionally.

Therefore, repeated concentration-response curves with increasing concentrations of ethanolic myrrh extract have been recorded and detailed analysis of the concentration-effect relation revealed that depending on the concentration both competitive and non-competitive elements contribute to the overall effect. For a better characterisation those elements were quantified and analysed separately. This elucidates that the competitive effects are mainly exerted in lower concentrations whereas non-competitive antagonistic behaviour is found in higher concentrations. Dual antagonism consisting of competitive and non-competitive elements is quite commonly found in biological drug testing. Especially in plant extracts these dual properties seem reasonable as a complex mixture of structurally related components contribute to the overall effect to a different extent and in varying modes of action. As previously mentioned Andersson and co-workers found that besides T-cadinol other sesquiterpenes exerted calcium antagonistic effects with varying potencies (Andersson *et al.* 1997). Moreover it is likely that especially in higher concentrations besides calcium channels other targets are influenced by terpenic or other compounds present in the ethanolic fraction besides the sesquiterpenes. It can be concluded from the data of the present study that ethanolic myrrh extract behaves mainly as a competitive antagonist on which, if higher concentrations are used a non-competitive inhibiting action is superimposed.

As previously pointed out, induced inflammation leads to a decreased motility of small intestinal tissue (Moreels *et al.* 2001; Demedts *et al.* 2006; Grossi *et al.* 1993). Moreover, it has been reported that calcium currents were suppressed upon inflammation (Akbarali *et al.* 2000; Kinoshita *et al.* 2003). Liu's group further showed that the current suppression was associated with a down regulated expression level of L-type calcium channels in membranes of circular smooth muscle cells of inflamed intestinal tissue (Liu *et al.* 2001). These observations could have an impact on the spasmolytic activity mediated by calcium channel blockade. The antispasmodic effects were therefore revised under inflamed conditions additionally.

Despite the reported inflammation-induced alterations of gastrointestinal contractility, the observed effects have overall been found to be comparable between healthy and inflamed conditions. Inhibition of ACh-induced contraction was demonstrated for both untreated and inflamed preparations. However, comparing the effect on induced contractions in inflamed and untreated tissue it must be stressed that the IC50 in inflamed tissue was slightly higher than in untreated tissue (277.4 µg/ml vs. 157.7 µg/ml). This can be explained by functional impairment of cholinergic neurotransmission as has been reported after induction of TNBS-mediated inflammation *in vivo* (Shi and Sarna 2000; Poli *et al.* 2001). The reported down-regulation of L-type calcium channels in inflamed intestinal tissue (Khan 1999) and subsequent impairment of calcium influx could yet be another possible explanation.

4.2.3 Anti-inflammatory activity of myrrh

The anti-inflammatory activity of myrrh was investigated in different inflammation models, whereby both the aqueous and the ethanolic extract were tested. In a first set of experiments the *in vitro* TNBS-inflammation model was used.

Aqueous myrrh extract (1 mg/ml) inhibited the TNBS-induced reduction of mucosal layer thickness. This observation is in accordance with results from the isometric tension measurement, where the aqueous myrrh extract (0.1 mg/ml) inhibited the decrease in contractility induced by TNBS. Yet, lower concentrations were able to antagonise the TNBS effect, whereby higher concentrations were not effective in this model. In line with these observations, the TNBS-induced overexpression of TNFα-mRNA, which is produced as response to a pro-inflammatory stimulus, was significantly reduced by intermediate concentrations of aqueous myrrh extract (1 and 10 mg/ml). This supports the assumption that the observed amelioration of TNBS-induced damages in morphology and contractility is due to anti-inflammatory effects.

Ethanolic myrrh extract (0.15 mg/ml) could not ameliorate the morphological damage induced by TNBS. Additionally, it could not antagonise the TNBS-induced decrease in contractility, yet

it induced a further decrease in ACh-induced contraction. Taking the demonstrated, very effective spasmolytic activity into consideration, it appears plausible, that the applied calcium channel blockade by components of the ethanolic myrrh extract for 30 minutes leads to a permanent decrease in ACh-induced contractions. This applies especially in high concentrations (1 mg/ml) since non-competitive antagonistic effects are dominating in this concentration range. A clear assumption regarding anti-inflammatory effects can therefore not be drawn using this model. Moreover, looking at the molecularbiological level, no statistically significant reduction of the TNBS-induced overexpression of TNFα-mRNA could be observed, yet the levels of TNFα gene expression after treatment with ethanolic myrrh extract were below those induced by TNBS.

To obtain a clear outcome about anti-inflammatory effects, is was necessary to use a simplified model without interferences by other pharmacological effects like spasmolytic activity. Therefore, macrophage cell lines and an LPS-inflammation model was used to investigate the production of pro-inflammatory mediators such as TNFα and nitric oxide (NO). Ethanolic myrrh extract inhibited the LPS-induced TNFα release from differentiated THP-1 cells concentration-dependently down to 2 % with an IC50 of 60.65 µg/ml. Cytotoxicity testing showed, that LDH release as a measure for necrotic cell death was not influenced in the concentrations tested. In the highest concentration tested (200 µg/ml) metabolic activity was reduced down to 48 %. The IC50 of metabolic activity in relation to the IC50 of TNFα release resulted in a selectivity index of 5.8, which means that a reduction of metabolic activity occurs in 6-fold higher concentrations than the inhibition of TNFα release. In accordance with this findings, ethanolic myrrh extract inhibited the LPS-induced NO release from murine macrophages (RAW 264.7 cells) down to 10 % with an IC50 of 24.24 µg/ml. It should be mentioned, that the murine system responds to inflammatory stimuli with a high production of NO in line with an overexpression of inducible NO synthase (iNOS) (Nathan 1992), whereas iNOS expression in human monocytes and macrophages has been difficult to demonstrate (Nussler *et al.* 1995; Denis 1991). These differences between species could explain the lower IC50 value for the NO-inhibition, since the murine system might respond more effectively to inhibitory effects of the ethanolic myrrh extract.

Aqueous myrrh extract exerted only in the highest concentration tested (1000 µg/ml) a moderate inhibitory effect on LPS-induced TNFα release from THP-1 cells and NO release from murine macrophages. Since only the highest concentration induced an effect, no conclusive concentration-response relation could be established.

Looking at the effects induced on basal TNFα or NO release, aqueous myrrh extract interestingly induced a release of TNFα or NO respectively in the highest concentration. These effects however occurred only in very high concentrations and are relatively weak compared

to those induced by LPS. Furthermore, neither LDH release nor metabolic activity of the cells was affected in this concentrations. Since related effects were observed in gene expression analysis, this issue will be discussed in that section.

Taken together, the molecularbiological effects, which could be investigated with the applied methods, suggest, that the anti-inflammatory effects of ethanolic myrrh extract are more pronounced than those induced by the aqueous extract. Since the functional and histological methods using isolated small intestinal preparations were not suitable to evaluate anti-inflammatory effects of the ethanolic extract due to spasmolytic activity, the anti-inflammatory effects could not be demonstrated in these methods.

However, for the aqueous myrrh extract, these functional and histological methods demonstrated an ameliorating effect of the TNBS-induced damage, which could be attributed to anti-inflammatory effects seen by analysis of TNFα gene expression in the intestinal tissue.

It was demonstrated, that the overall anti-inflammatory effect of myrrh is mediated through suppression of TNFα and NO release. The findings are in accordance with results from other studies where an aqueous myrrh extract reduced LPS-induced TNFα, NO and prostaglandin E2 (PGE2) release in peritoneal macrophages (Kim *et al.* 2012). In this study the aqueous extract was obtained by extracting powdered myrrh in boiling water for two hours, without previous removal of the ethanol-soluble resin fraction. This could explain the fact, that this aqueous extract reduced pro-inflammatory cytokine production, whereas the aqueous extract of the present study did not. Remaining components of the ethanol-soluble resin which were dissolved in water under the extracting conditions of Kim *et al.* could have induced the anti-inflammatory effect. These compound would appear in the ethanolic extract used in this study, which is in accordance with the obtained results. Several animal studies confirmed the anti-inflammatory property of myrrh, whereby it reduced the growth of carrageenan or formaldehyde induced oedemas and decreased prostaglandin E2 or NO release in inflamed tissue (Su *et al.* 2011; Su *et al.* 2012; Tariq *et al.* 1986; Atta and Alkofahi 1998).

Microarray gene expression analysis

To determine its influence on the gene expression profile of stimulated native human macrophages, ethanolic myrrh extract was applied to the cells simultaneously with LPS/IFNγ for 24 hours. For this experiments a concentration of 30 µg/ml was selected based on previous studies and preliminary flow cytometry experiments.

Ethanolic myrrh extract led to more than 10-fold up-regulation of 84 genes and more than 10-fold down-regulation of 202 genes compared to a single LPS/IFNγ-treatment. Genes that were affected could be clustered in significantly enriched GO-categories belonging to the molecular function of oxidoreductase activity. However, oxidoreductases are enzymes, which catalyse

redox reactions and their functioning belongs to basic cell biological processes which cannot clearly be attributed to anti-inflammatory pathways.

Aqueous myrrh extract (500 µg/ml) had no major influence on the gene expression profile of LPS/IFNγ stimulated native human macrophages. Only a number of 25 genes was up-regulated more than 10-fold and a number of 112 genes was down-regulated more than 10-fold compared to a single stimulation with LPS/IFNγ. Neither of those genes could be clustered in a significantly enriched GO-category.

However, in line with findings made in the LPS-inflammation model in human and murine macrophages, single treatment with aqueous myrrh extract influenced the basal gene expression of the native human macrophages. Compared to untreated control, single treatment with aqueous myrrh extract led to a more than 10-fold up-regulation of 678 genes of which 20 are up-regulated more than 100-fold and one gene is up-regulated more than 1000-fold. Gene ontological enrichment analysis revealed, that a significant number of those genes, that were up-regulated more than 10-fold, can be associated with immune response such as chemokine activity and cytokine receptor activity. On the other hand, genes that were down-regulated more than 10-fold can be associated with mitosis and cell cycle regulation. This suggests, that components of aqueous myrrh extract itself trigger an inflammatory response since similar effects could be observed after single LPS/IFNγ-stimulation. This observation is in accordance with earlier findings of an induced TNFα-production in THP-1 cells and an induced NO release in murine macrophages upon incubation with aqueous myrrh extract. However, it was seen in the methods testing TNFα and NO release, that the stimulating effect was far lower compared to that induced by LPS in a concentration of 100 ng/ml.

An initial consideration suggests the possibility of a contamination of the extract with lipopolysaccharides. However, a LAL endotoxin assay reported no endotoxin contamination of the extract in the concentrations tested.

Another possibility is, that water-soluble myrrh components in the extract induce these effects. The water soluble gum fraction of powdered myrrh is composed of a heterodisperse mixture of proteoglycans with a dominating amount of uranic acid rich compounds (Wiendl *et al.* 1995). It is possible that among those polysaccharides structures can be found, which trigger immune response for example by activating certain pattern recognition mechanisms. Similarly, proteogylcans from mushrooms like *Phellinus linteus* (Hymenochaetaceae) are known to induce macrophage activation via toll-like receptor 2 and/or toll-like receptor 4 mediated pathways (Kim *et al.* 2004).

4.3 Coffee charcoal

4.3.1 Extract characteristics

Coffee charcoal is described as the milled, roasted to blackening outer seed parts of green dried *Coffea Arabica* L. fruits. Kuhn and Schäfer (1939) have analysed the ingredients of coffee charcoal in comparison to green coffee. Depending on the roasting degree, the powdered substance contains 0.8 to 1.0 % caffeine which correlates to 75 % of the content in roasted coffee. The chlorogenic acid concentration is about 1.89 %, the caffeic acid concentration about 0.31 % and the trigonelline concentration with 0.29 % approximately half of the content in roasted coffee (Kuhn and Schäfer 1939). Examination of the absorption capacity showed an increase with rising roasting times. Coffee charcoal (1.0 g) binds about 50 mg methylene blue, whereas green coffee was able to bind only 9.0 mg. However, the absorption capacity of activated charcoal was not achieved (Kuhn and Schäfer 1939).

For the pharmacological experiments in this study a filtrated aqueous extract was used, which therefore contained water-soluble substances like caffeine, chlorogenic acid and trigonelline. Diterpenes like kahweol and cafestol were partially removed by the filtration process.

4.3.2 Spasmolytic activity of coffee charcoal

Coffee charcoal is traditionally used as an antidiarrheal whereby typically an average daily dose of 9.0 g is recommended by the Commission E. For this indication the monograph refers to its absorbent and astringent properties, which mediate the therapeutic effect similar to activated charcoal.

The spasmolytic activity of an aqueous coffee charcoal extract, which could contribute to an antidiarrheal effect, was examined in the present study and no influence on ACh-induced contraction was found. Additionally no effect on basal muscular tone was found in preliminary experiments (results are not presented).

The effect of coffee on gastrointestinal motor function is discussed controversially. *In vivo* studies indicated an increase in colonic motor activity (Brown *et al.* 1990; Rao *et al.* 1998), whereas no clear effect on gastric motor function was found (Chan *et al.* 2000; Boekema *et al.* 2000). However, because of a latency time of several minutes it appears that those *in vivo* effects are mediated by neural mechanisms or gastrointestinal hormones and no direct action on the colon occurs. Some older *in vitro* studies, performed with instant and decaffeinated coffee, report cholinomimetic or muscarinic actions of some coffee compounds and a relaxing activity for caffeine particularly (Tse 1992; Atkinson 1976), however clear evidence for these reported effects is lacking to date.

It is likely, that the process of overroasting in the manufacturing process of coffee charcoal has destroyed potential agents and the final mass ratio of those compounds in the powdered substance is too low to mediate any pharmacological effects.

Moreover, the information provided by the *in vitro* study performed in the present study using isolated organs is limited, since indirect effects, mediated by neuronal or gastrointestinal messengers are neglected.

4.3.3 Anti-inflammatory activity of coffee charcoal

The investigation of anti-inflammatory activity of an aqueous extract from coffee charcoal, which is processed in Myrrhinil-Intest® was conducted using the TNBS- and the LPS-inflammation model in isolated small intestinal preparations and human macrophage like cells respectively.

In the TNBS-inflammation model no anti-inflammatory effect was observed. Coffee charcoal did not influence the TNBS-induced morphological damage or the contractility decrease of the small intestinal preparations. Furthermore, no influence was detected on the TNBS-induced overexpression of TNFα-mRNA.

Investigations on the LPS-induced TNFα release from differentiated THP-1 cells revealed an inhibiting effect by coffee charcoal in higher concentrations. TNFα release was suppressed down to 56 % compared to single LPS-stimulation. However, the IC50 value was comparably high (IC50 = 1886 µg/ml) and the maximal inhibitory effect was much less pronounced than the effect induced by the glucocorticoid budesonide, which was used as positive control.

A potential active compound could be caffeic acid and its quinic acid-ester chlorogenic acid, whose anti-inflammatory activities have been described earlier (Koshihara *et al.* 1984). Mechanisms found to be involved include the inhibition of phosphorylase kinase and protein kinase A and C (Nardini *et al.* 2000) as well as suppression of NF-κB and MAPKs (Feng *et al.* 2005). Furthermore it has been reported, that caffeic acid directly suppresses interleukin-1 receptor-associated kinase 1 and 4 (IRAK1 and IRAK4) which are involved in downstream toll-like receptor 4 signalling (Yang *et al.* 2013).

Microarray gene expression analysis

Gene expression analysis was performed to assess the influence of coffee charcoal extract on the gene expression profile of stimulated human macrophages. Therefore, a concentration of 500 µg/ml was chosen based on previous cell culture studies and preliminary flow cytometry experiments.

Coffee charcoal extract (500 µg/ml) exerted the strongest effects on LPS/IFNγ-induced gene up-regulation. It suppressed the LPS/IFNγ-induced expression of the genes CXCL13,

CXCL10 and CXCL11 more than 100-fold. A number of 8 genes was suppressed more than 10-fold. It appears noteworthy, that most of those genes are associated with immune response, in particular with chemokine signalling. On the other hand, coffee charcoal extract led to an up-regulation of 195 genes by more than 10-fold compared to LPS/IFNγ-stimulation. Those genes can be clustered in significantly enriched categories associated with cell motility such as cell migration and leukocyte migration and cellular response to corticosteroid stimulus. This suggests, that coffee charcoal interferes with the chemotactic response of activated macrophages and thereby influences inflammatory response.

This observation is in accordance with an *in vivo* study investigating the effect of coffee consumption on the immune system which reported, that the chemotaxis activity of mononuclear cells was increased in test persons after coffee consumption (Melamed *et al.* 1990).

Adenosine is known to promote neutrophil chemotaxis by activating adenosine receptors (Rose 1988). Caffeine as a non-selective adenosine receptor antagonist (Fredholm *et al.* 1999) could therefore be an active substance responsible for influencing the chemotactic response of coffee charcoal. Rose was able to show in his study, that the antagonist NECA (5′-(N-ethylcarboxamido)adenosine) completely reversed the agonist-induced enhancement of chemotaxis but did not affect chemotaxis by itself (Rose 1988). Likewise, caffeine could be responsible for the inhibition of the LPS/IFNγ-induced chemotactic response in macrophages. However, these considerations have to be reinforced in respective studies.

4.4 Conclusion

Several clinical studies showed that the fixed combination Myrrhinil-Intest® - containing myrrh, chamomile flower and coffee charcoal - is a potent agent against gastrointestinal disorders (Langhorst *et al.* 2013; Lühr 1996; Thilo-Körner 2006). The therapeutic areas for which Myrrhinil-Intest® is applied in medicinal practice, covers various gastrointestinal disorders such as chronic diarrhea, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).

For the investigation of pharmacological effects, spasmolytic and anti-inflammatory activities were of interest since mainly these pharmacological effects seem to contribute to the reported therapeutic effectiveness.

The spasmolytic activity is mainly driven by ethanolic myrrh extract, whose calcium antagonistic effects led to a relaxation of the smooth muscle in small intestinal preparations. It can be assumed, that *in vivo* myrrh acts locally at the muscle cells of the intestine and no absorption of the active ingredients into the circulatory system occurs. Adverse events that would follow a systemic action can be derived from the action of therapeutically applied calcium

channel blockers for the treatment of cardiac diseases and include bradycardia, low blood pressure and reflex tachycardia amongst others. However, in a study conducted by Langhorst and co-workers in which 47 patients were treated with Myrrhinil-Intest® (four tablets three times daily) for a 12-months period, no cardiovascular adverse events were observed (Langhorst *et al.* 2013). This indicates, that the systemic absorption rate of the active ingredients, which are responsible for calcium antagonistic effects, is too low to cause cardiovascular effects.

The spasmolytic activity of chamomile flower certainly contributes towards the overall effect of the fixed combination. Looking at the ability to decrease ACh-induced contractions, chamomile flower is somewhat less effective than myrrh, since their IC50 values are comparable, yet myrrh induced a stronger maximal inhibitory effect. However, a synergistic effect between these two agents cannot be ruled out.

An *in vivo* study performed by Kanazawa for the treatment of inflammatory bowel syndrome indicated, that pain hypersensitivity and an enhanced colonic motility, which is characterised by increased phasic motility and enhanced smooth muscle tone, are independent factors contributing to symptom severity of IBS patients (Kanazawa *et al.* 2008). Effective treatment, which addresses one or both factors contributes therefore to the improvement of the overall symptom complex. In this context, the application of Myrrhinil-Intest® for the treatment of not only diarrheal symptoms but also IBS in general becomes plausible because of the antispasmodic effects of its components. However, treatment of multifactorial disorders such as IBS should not be based on only one strategy but provide a multi-target approach as it is the case for many herbal therapies.

Inflammation is another factor which has been found to play a crucial role in the pathophysiology of IBS. Persisting inflammatory processes can lead to a number of functional and structural alterations in the concerned tissue impairing contractility and mucosal barrier function. It is well established that inflammation markedly alters motility patterns of the gastrointestinal tract. Phasic and tonic contractions have been shown to be suppressed, whereas the giant migration peristaltic contractions of large amplitude appear to be increased in frequency generating diarrheal symptoms associated with inflammation (Malykhina and Akbarali 2004).

In this regard, the anti-inflammatory activity of Myrrhinil-Intest® could be another strategy to improve symptom severity of functional bowel disorders. The anti-inflammatory activity of chamomile flower is well established and was reinforced with the extract used in Myrrhinil-Intest® and the methods applied in this study. The chamomile flower extract has a pronounced anti-inflammatory potential which could be observed in the TNBS-inflammation

model using isolated small intestinal preparations but also in the LPS-inflammation model using human macrophage like cells.

The anti-inflammatory potential of myrrh is more complex and components of the aqueous as well as the ethanolic extract seem to contribute to the effects. Ethanolic myrrh extract exerts a very distinct anti-inflammatory effect in cell culture models with an IC50 ranging from 24 to 61 µg/ml. Aqueous myrrh extract however ameliorated the damaging effects of the pro-inflammatory TNBS on intestinal tissue. It is likely, that both effects are exerted via different mechanisms which could explain the diverse response rates in the different models used.

Coffee charcoal did not exert distinct anti-inflammatory effects in the TNBS-inflammation model. Using the LPS-inflammation model in macrophage like cells, a decreasing activity on TNFα release was observed in high concentrations (IC50 = 1886 µg/ml). These effects are likely to be unspecific due to the high half maximal inhibitory concentration and in comparison to those induced by myrrh and chamomile of minor significance. However, other pharmacological activities could contribute to the therapeutic potential of Myrrhinil-Intest®. It was observed in gene expression analysis, that especially the LPS-induced elevated chemokine signalling was influenced by coffee charcoal extract. The clinical significance of this observation is yet to be questioned. Its absorbent and adstringent properties certainly contribute mainly to the overall effectiveness of the fixed combination.

Taken together, it could be demonstrated, that the components of Myrrhinil-Intest® are able to target gastrointestinal disorders by exerting spasmolytic and anti-inflammatory effects. These pharmacological effects provide plausibility of the reported clinical effectiveness.

Further investigations could focus on the identification of active substances within the herbal preparations, which are responsible for the spasmolytic and anti-inflammatory effects. This knowledge would be beneficial for a future standardisation of the herbal ingredients. Therefore, bioguided fractionation should be performed to identify active principles. On the same account, it is necessary to perform a phytochemical characterisation of all extracts used in Myrrhinil-Intest®. Furthermore, combination analysis of the extracts could be undertaken to identify synergistic effects between the herbal ingredients especially in the field of spasmolytic activity with chamomile and myrrh, but also for the anti-inflammatory effects. Evidence for synergistic effects among the herbal ingredients would for instance reinforce the superiority of this combination product over a mono-component product.

5 Summary

The fixed herbal combination Myrrhinil-Intest®, which consists of myrrh, chamomile flower and coffee charcoal is marketed in Germany since 1959 and applied in medical practice for the treatment of gastrointestinal disorders such as functional diarrhea, irritable bowel syndrome and inflammatory bowel diseases. Myrrh is described as the oleo-gum resin from mainly *Commiphora molmol* Engler (Burseraceae), coffee charcoal are the powdered outer seed parts of green dried *Coffea Arabica* L. (Rubiaceae) fruits, which are roasted to blackening and chamomile flowers are the flower heads of *Matricaria recutita* L. (Asteraceae).

The clinical effectiveness of Myrrhinil-Intest® for the treatment of various gastrointestinal disorders was demonstrated in several clinical studies and is described in various experience reports.

Since market release in 1959, the regulatory requirements for herbal medicinal products have underwent major changes. Following the establishment of the German Medicines Act in 1976, which introduced a definition of medicinal products, manufacturers were obliged to provide proof of quality for the medicinal product, whereby safety and efficacy for in principle well-known constituents of herbal medicinal products could be demonstrated by the Commission E. Myrrhinil-Intest® obtained a subsequent approval within this regulatory context in 2001.

In the course of further harmonisation across the European Union the Directive on Traditional Herbal Medicinal Products (Directive 2004/24/EC) introduced a regulatory framework for herbal medicinal products which allows a registration as “traditional herbal medicinal product” or marketing authorisation for well-established medicinal products. Within this context, the regulatory status for Myrrhinil-Intest® had to be adapted to a traditional use registration and new documentation in line with the requirements from the directive specified in § 16a has to be provided. One aspect within these requirements concerning pharmacological proof, asks for "plausibility of pharmacological effects or efficacy of the medicinal product on the basis of long-standing use and experience" (Directive 2004/24/EC § 16a). This knowledge has to be reinforced with the provision of supportive preclinical data.

To address the increasing need for robust preclinical data, aim of the present study was to update the pharmacological profile of Myrrhinil-Intest® with the provision of pharmacological data obtained with the means of modern biochemical and pharmacological methods. In this context the spasmolytic and anti-inflammatory activities were of interest since mainly these pharmacological effects seem to contribute to the reported therapeutic effectiveness.

For the experiments, extracts of myrrh and coffee charcoal were prepared to obtain suitable testing agents. The dried extract of chamomile flower, which is used in the finished dosage

form was ready to use by dilution in ethanol or DMSO. Coffee charcoal was extracted with water to obtain an aqueous extract. The water and the ethanol soluble fraction of myrrh was separated by preparing an aqueous and an ethanolic extract.

Spasmolytic activity was assessed using isolated rat small intestinal preparations. Functional investigations were carried out measuring isometric contractions.

Myrrh and chamomile flower exerted a spasmolytic effect, whereas coffee charcoal induced no effect. The spasmolytic activity of myrrh is more pronounced and mediated by inhibition of L-type calcium channels, whereby competitive inhibition occurs in lower concentration on which non-competitive effects in higher concentration are superimposed.

To examine anti-inflammatory activity an *in vitro* TNBS-inflammation model was used. Inflammation was induced in rat small intestinal preparations and the influence of the extracts on contractility, morphology and TNFα-mRNA expression was detected. These parameters were measured using isometric tension measurement, histological analysis and TNFα gene expression analysis respectively.

Furthermore, a LPS-inflammation model was utilised. Thus, LPS was used as a pro-inflammatory stimulus to activate macrophages or macrophage like cells and the effect of the extracts on cytokine signalling was assessed.

Chamomile flower induced a coherent anti-inflammatory effect which was detectable in both the TNBS- and the LPS-inflammation model.

The anti-inflammatory potential of myrrh provides a complex picture. The aqueous fraction of myrrh induced anti-inflammatory effects, which could be observed in the TNBS-inflammation model. It ameliorated TNBS-induced contractility decrease, morphological damage and overexpression of TNFα-mRNA. However, it had no influence on LPS-induced TNFα release from macrophage like cells.

The ethanolic fraction induced a very pronounced effect in the LPS-inflammation model by inhibiting LPS-induced TNFα and NO release. Yet, no effect could be observed in the TNBS-inflammation model.

The anti-inflammatory activity of coffee charcoal is of minor potency as it inhibited the LPS-induced TNFα release only in higher concentrations.

In order to obtain an insight in the mechanisms underlying any anti-inflammatory activity, gene expression analysis was performed. Thus, the influence of the extracts on the gene expression

profile of native human macrophages was studied. A LPS/IFNγ mixture was used as pro-inflammatory stimulus.

Chamomile flower and coffee charcoal markedly influenced the gene expression profile. Especially coffee charcoal inhibited the LPS/IFNγ-induced expression of genes associated with chemokine signalling. Ethanolic myrrh extract did not explicitly influence the expression of genes that can be associated with inflammatory processes. However, single treatment with aqueous myrrh extract resulted in a change in gene expression profile similar to this induced by LPS/IFNγ, indicating an immunostimulatory action.

The presented study demonstrates the pharmacological potential of the herbal ingredients of the fixed combination with respect to its spasmolytic and anti-inflammatory activities. Taken together, it could be demonstrated, that the components of the herbal combination are able to target gastrointestinal disorders by exerting spasmolytic and anti-inflammatory effects which reinforce the reported clinical effectiveness.

Zusammenfassung

Das traditionelle pflanzliche Arzneimittel Myrrhinil-Intest® - bestehend aus Myrrhe, Kamillenblüten und Kaffeekohle - wird seit 1959 in Deutschland vermarktet. Sein Anwendungsgebiet umfasst gastrointestinale Beschwerden wie zum Beispiel funktionelle Diarrhoe, Reizdarmsyndrom und chronisch entzündliche Darmerkrankungen.

Myrrhe bezeichnet das an der Luft gehärtete Gummiharz von hauptsächlich *Commiphora molmol* Engler (Burseraceae). Kamillenblüten sind die Blütenköpfe von *Matricaria recutita* Linné (Asteraceae) und Kaffeekohle wird durch Überröstung von grünen getrockneten Kaffeefrüchten (*Coffea Arabica* Linné, Rubiaceae) gewonnen.

Die klinische Wirksamkeit des Präparates für die Behandlung von verschiedenen gastrointestinalen Beschwerden wurde in mehreren klinischen Untersuchungen und Erfahrungsberichten belegt.

Seit der Markteinführung 1959 haben sich die regulatorischen Anforderungen an pflanzliche Arzneimittel gewandelt. Nach der Einführung des Arzneimittelbegriffes mit der Etablierung des Arzneimittelgesetzes in Deutschland (1976) oblag es den Arzneimittelherstellern, Belege zur Qualität ihres Produktes zu erbringen. Hingegen war es möglich, Unbedenklichkeit und Wirksamkeit eines pflanzlichen Arzneimittels mit vom Grundsatz her bekannten Stoffen anhand einer Aufbreitungsmonografie der Kommission E zu belegen. Myrrhinil-Intest® erlangte im Jahr 2001 im Rahmen dieser Gesetzgebung eine sogenannte Nachzulassung.

Im Zuge eines Harmonisierungsprozesses innerhalb der Europäischen Union führte die Richtlinie über pflanzliche Arzneimittel (Richtlinie 2004/24/EG) eine Gesetzgebung für traditionelle pflanzliche Arzneimittel ein, welche neben der Zulassung als Arzneimittel mit vom Grundsatz her bekannten Stoffen auch eine Registrierung als sogenanntes „traditionelles pflanzliches Arzneimittel“ ermöglicht. Innerhalb dieser Gesetzgebung wurde der regulatorische Status für Myrrhinil-Intest® angepasst und eine Registrierung angestrebt, woraufhin Belege gemäß der Anforderungen der Richtlinie zur Verfügung gestellt werden müssen. Ein Aspekt dieser Anforderungen umfasst Belege zur Plausibilität der pharmakologischen Wirkung oder Wirksamkeit (Richtlinie 2004/24/EG § 16a). Begleitend hierzu müssen diese Informationen durch robuste präklinische Daten untermauert werden.

Ziel der vorliegenden Arbeit war es, das pharmakologische Profil des pflanzlichen Arzneimittels Myrrhinil-Intest® zu aktualisieren, indem die pharmakologische Wirkung der Einzelkomponenten mit Hilfe biochemischer und pharmakologischer Methoden untersucht wird.

In diesem Zusammenhang sollten im Einzelnen die spasmolytischen und anti-inflammatorischen Wirkungen der Komponenten untersucht werden, da diese hauptsächlich zu den beschriebenen klinischen Effekten beizutragen scheinen.

Für die Durchführung der Experimente wurden Auszüge aus Myrrhe und Kaffeekohle hergestellt, um applikationsfähige Testsubstanzen zu erhalten. Der Kamillenblüten-Trockenextrakt, welcher in der fertigen Darreichungsform verwendet wird, ist nach Lösung in Ethanol oder DMSO gebrauchsfertig. Ein wässriger Kaffeekohle-Auszug wurde nach Extraktion mit gereinigtem Wasser und anschließender Lyophilisation hergestellt. Durch Extraktion mit gereinigtem Wasser bzw. Ethanol und anschließender Lyophilisation wurden die wasser- bzw. ethanollöslichen Fraktionen des Myrrheharzes getrennt und ein wässriger sowie ein ethanolischer Myrrhe-Extrakt hergestellt.

Die spasmolytische Aktivität wurde an isolierten Dünndarmpräparaten der Ratte unter Verwendung isometrischer Kontraktionsmessungen untersucht.

Myrrheharz und Kamillenblüten zeigten spasmolytische Aktivität, während Kaffeekohle keinen spasmolytischen Effekt induzierte. Die spasmolytische Wirkung von Myrrhe ist stark ausgeprägt und wird durch eine Hemmung von L-Typ Calciumkanälen vermittelt. Hierbei treten kompetitive antagonistische Effekte in niedrigeren Konzentrationen auf, welche in höheren Konzentrationen durch nicht-kompetitive antagonistische Effekte überlagert werden.

Für die Untersuchungen zur anti-inflammatorischen Aktivität wurde ein TNBS-Entzündungsmodell genutzt. Die Entzündung wurde *in vitro* in isolierten Dünndarmpräparaten der Ratte induziert und der Einfluss der Pflanzenextrakte auf Kontraktilität, Morphologie und TNFα-mRNA-Expression untersucht. Diese Parameter wurden unter Verwendung isometrischer Kontraktionsmessung, histologischer Untersuchungen bzw. TNFα-Genexpressionsanalyse bestimmt.

Weiterführend wurde ein LPS-Entzündungsmodell verwendet. Hierbei wurde LPS als pro-inflammatorischer Stimulus genutzt, um Makrophagen bzw. Makrophagenähnliche zu aktivieren und anschließend den Effekt der Extrakte auf die Zytokinausschüttung zu untersuchen.

Kamillenblüten-Extrakt induzierte einen deutlichen anti-inflammatorischen Effekt, welcher sowohl im TNBS- als auch im LPS-Entzündungsmodell sichtbar war.

Die anti-inflammatorische Aktivität von Myrrheharz bietet ein komplexeres Bild. Der wässrige Myrrhe-Extrakt zeigte im TNBS-Entzündungsmodell anti-inflammatorische Effekte. Er hemmte

den TNBS-induzierten Kontraktilitätsverlust sowie die morphologische Schädigung und Überexpression von TNFα-mRNA. Auf die LPS-induzierte Freisetzung von TNFα aus makrophagenähnlichen Zellen hatte er jedoch keinen Einfluss.

Der ethanolische Myrrhe-Extrakt führte zu einer deutlichen Verminderung der LPS-induzierten Freisetzung von TNFα und Stickstoffmonoxid. Im TNBS-Entzündungsmodell konnte jedoch kein Effekt beobachtet werden.

Die anti-inflammatorische Aktivität von Kaffeekohle in den verwendeten Modellen ist von geringerer Wirkkraft. Lediglich im LPS-Entzündungsmodell wurde in höheren Konzentration die LPS-induzierte Freisetzung von TNFα vermindert.

Um nähere Informationen zu den zugrundeliegenden Mechanismen der beobachteten Effekte zu erhalten, wurde eine Mikroarray-Genexpressions-Analyse durchgeführt. Hierbei wurde der Effekt der Pflanzenextrakte auf das Genexpressionsprofil nativer humaner Makrophagen untersucht. Als pro-inflammatorische Stimuli wurden LPS und Interferon-y (IFNγ) verwendet.

Kamillenblüten und Kaffeekohle beeinflussten das Genexpressionsprofil unter LPS/IFNγ-Stimulation deutlich. Dagegen führte weder ethanolischer noch wässriger Myrrhe-Extrakt unter LPS/IFNγ-Stimulation zu einer deutlichen Beeinflussung der Expression von Genen, die mit Entzündungsprozessen in Verbindung stehen. Wässriger Myrrhe-Extrakt führte jedoch an unstimulierten Zellen zu einer vergleichbaren Veränderung des Genexpressionsprofils wie nach LPS/IFNγ-Stimulation, was als Hinweis auf einen immunstimulierenden Effekt gewertet werden kann.

Die vorliegenden Untersuchungen zeigen das pharmakologische Potenzial der Komponenten des pflanzlichen Arzneimittels in Bezug auf ihre spasmolytische und anti-inflammatorische Aktivität. Es konnte nachgewiesen werden, dass die pflanzlichen Inhaltsstoffe spasmolytische und anti-inflammatorische Effekte induzieren, welche für die Behandlung von gastrointestinalen Beschwerden von Bedeutung sind und die beschriebene klinische Wirksamkeit des Präparates untermauern.

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Acknowledgement

I would like to extend my gratitude to the many people who helped and supported me and without whom this work would not have been possible.

My first and sincere appreciation goes to my supervisor Prof. Dr. Karen Nieber for supporting my scientific progress during the past years. I would like to thank her in particular for providing the opportunity to work on this interesting project and the numerous pieces of useful advice and valuable suggestions. Also I am very grateful for the many opportunities to present my results on national and international congresses and the possibilities to discuss them with professional audience throughout the years.

I am grateful to all members of the former workgroup of Prof. Nieber at the Institute of Pharmacy (University of Leipzig) for the great working atmosphere, the fruitful and very valuable discussions but also and simply for the good times in Leipzig and beyond.

Moreover I wish to express my gratitude to Prof. Dr. Jürgen Arnhold and his group at the Institute for Medical Physics and Biophysics (University of Leipzig) for giving me the opportunity to continue working on this very interesting project and the ongoing scientific guidance.

Also I thank all staff members of the Faculty of Biosciences, Pharmacy and Psychology of the University of Leipzig and especially those of the Institutes of Pharmacy, Biology and Biochemistry for the pleasant working atmosphere and helpful advices whenever needed. Particularly I would like to thank Romina Kühne and Mrs. Baumbach (Department Cell and Developmental Biology, University of Leipzig) for the introduction into histological techniques and the helpful advices for the work with the THP-1 cells.

My sincere gratitude goes to Prof. Dr. Andreas Hensel and his group at the Institute of Pharmaceutical Biology and Phytochemistry (Universtiy of Münster) for the opportunity to conduct the cellular biological experiments with the RAW 264.7 macrophages. I enjoyed the trip to Münster and the work in his laboratories very much and my special thank goes to Jana Marina Schmuch for the training and her huge efforts in assisting me with my studies throughout my stay.

Finally, I want to express my deep gratitude to my family and my dearest friends for the faith they had in me and the unreserved support they gave me during the last years to make this happen.

Leipzig, 25th September 2014

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